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13. ABSTRACT ***(Maximum 200 Words)***

The deadly consequences of breast cancer are due to metastasis, a process in which tumor cells penetrate the blood vessels and enter other tissues to spread the cancer. This movement through vessels and tissues is attributed to a group of digestive enzymes (the matrix metalloproteinases or MMPs) that can destroy the matrix in advance of tumor cell movement. These MMPs are normally produced in small amount and are held in check by inhibitors in the tissues (tissue inhibitors of MMPs or TIMPs). We took 160 samples of breast tissues (benign tumors and various carcinomas) and measured the production of six different MMPs and 2 TIMPs in a unified multipronged approach. We used antibody methods to see which cells are producing these enzymes and inhibitors. The most prominent enzyme was MMP-9, also known as gelatinase B, which is able to break down the wall that forms around tumor cell clusters. While this, and other MMPs were elevated in cancer, the TIMP inhibitors were produced at levels well below normal. This results in an imbalance in which the destructive proteases greatly outweigh the controlling inhibitors, facilitating the spread of the cancer. Biochemical quantification of MMPs by zymography showed an overall increase in all types of MMPs in cancer tissues. MMP-9 was the key MMP; it was present at levels 0.39 (*in situ* ductal) to 4.8 (infiltrating ductal) µg/g wet weight tissue in cancer tissues compared to unquantifiable amounts in normal (3/6) and benign neoplasm (25/30) tissues. Zymography also showed a fraction of MMP-9 and MMP-2 in their active forms in high grade breast cancers compared to normal and benign tissues Reverse zymography showed the presence of TIMPs -1, -2 and -3 in all breast tissues. Quantitation by TIMP-1 and TIMP-2 ELISA kit clearly showed that total amounts of TIMPs were lower in cancer (1.7 µg/g wet weight tissue in adenocacinomas compared to higher amounts in normal (5.8µg) and benign (5.6µg) tissues. The production of TIMPs in cancer tissues falls far short of the amount needed to counteract the excessive production of MMPs leading to an imbalance in enzyme and inhibitor expression. Quantitation of MMP and TIMP gene expresion (MMP-9, -2, -1, TIMP-1 and -2) by RT-PCR method. are in agreement with both zymography and immunohistochemical findings.

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(5) INTRODUCTION

It is widely accepted that invasiveness and metastasis by human cancers depend on degradation by specific group of matrix metalloproteinases (MMPs) of the basement membranes, collagen fibers and other components that would otherwise block the spread of developing tumors [1-4]. Metastasis of an initially localised tumor to vital organs [5,6] involve very many complex biochemical and cellular events [7,8]. The tumor cells attach to the extracellular matrix (ECM), penetrate into and through the basement membrane around the vessels and then re-enter into the stroma to establish a new secondary tumor [9,10]. The breakdown of the ECM can be traced to the action of one or more members of the family of zinc proteases named **matrix metalloproteinases (MMPs)** secreted as proenzymes [11,12] and activated by other proteases outside the cell [11]. There are seventeen different MMPs, however, the degradation of ECM [13,14] has been attributed to the action of gelatinases: 72 kDa gelatinase (gelatinase A, type IV collagenase, MMP-2) [15] and 92 kDa gelatinase (gelatinase B, type V collagenase, MMP-9) and were implicated in human breast cancer [16,17]. Metalloproteinase action is limited by specific tissue inhibitors, TIMPs and they play an important role in controlling ECM degradation. Four species, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 [10,12,18,19] neutralize the destructive activities of MMPs. Since normal cells as well as non-invasive cells produce MMPs and TIMPs, it seems likely that the extent of activation of MMPs and the levels of inhibitory TIMPs may be two key factors in the progression of normal to invasive cell type.

(6) BODY

The underlying hypothesis to be tested was that an imbalance occurs when invasive cells produce (or cause to be produced) more active enzymes and less inhibitors than normal cells or cells of benign tumors.

In order to test the hypothesis, the following types of studies were planned. i) Identification and localization of various MMPs and TIMPs by immunohistochemistry by the use of specific anti-MMPs and anti-TIMPs IgGs. ii) Determination of the amounts and ratios of active and latent enzyme by substrate zymography and immuno-precipitation combined with zymography. iii) Determination of mRNA levels (Northern blotting or RT-PCR analysis) for the MMPs and TIMPs in the same tissue samples. iv) Culture of epithelial cells and fibroblastic cells growing out from explants of human mammary carcinomas and determination of their production of active and latent MMPs and TIMPs.

The above studies were described and outlined in the original statement of work (SOW) and conducted very close to the time period of four years stated in SOW. The totals samples processed each year were also according to those outlined in SOW. Over two hundred samples were analyzed, however, 160 breast samples were included in the evaluation of the MMPs and TIMPs. The explant culture studies are on going to at least obtain results from six fresh breast tissues.

The sudy was nearly completed early 1999. Only the RT-PCR analysis for MMP-13 and MMP-1 were delayed. The study also generated numerous amount of data that needed to be collated. The data presented below follow the guidelines of the journal format of American Journal of Pathology. This includes the Materials and Methods section. It is not yet decided to submit the multipronged study in a single manuscript or submit the findings as two manuscripts. One would be to interrelate the zymographical and immunohistochemical evaluations of breast samples and the second manuscript relating biochemical and mRNA levels by molecular biological evaluations (RT-PCR analysis) of related breast tissue samples. The results are presented below adhering to SOW and to the guidelines of the journal format,

MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN HUMAN BREAST ADENOCARCINOMA

Role of Gelatinase B in the metastatic progression of breast tumor

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Materials and Methods

Obtaining Human Tissues:

The tissues (~ 1 cm^3, snap frozen, stored at -70^o C) and diagnosed as displaying breast carcinoma together with normal or benign breast tissues were obtained from the Tissue Procurrement Laboratory, Pathology Department, University of Miami Medical School, Miami Florida. The obtained tissues were in accordance with the University of Miami's Human Subjects Institutional Review Board. To obtain meaningful and stastically viable evaluations of the anlyses, focus was based on the availability of the reasonable number of a particular type of biopsied breast tissues. These included tissues showing normal category, benign disorders (fibroadenoma and fibrocystic changes) and noninfiltrating or infiltrating (invasive) breast carcinomas. Pathology reports showing these categories and others were obtained and carefully evaluated by Dr. Sittler. Breast samples were coded and then processed for various biochemical, immunohistochemical and molecular biological analyses. The Pahtology reports showed both the surgeon's diagnosis as well as the Pathologists conclusive findings.

Pathology of Breast Tissues

Normal breast consists of epithelial structures in the form of ducts and lobules embedded in a fibrofatty stroma. Fibrocystic changes occur in the breast with an increase in fibrous stroma associated with the dilatation of ducts to form cysts of various sizes some of which show apocrine type metaplasia. In addition there are various amounts of epithelial hyperplasia seen in fibrocystic change ranging from mild to marked with cytologic atypia. Tumors of the breast may arise from either the epithelial component or the stroma and these lesions are of greater clinical concern than non-neoplastic breast tissue.. These changes are nonproliferative lesions (benign disorders)with mild hyperplasia of epithelial cells within a duct or two. The breast tumors are the lesions of the breast and arise from connective tissue or epithelial structures and

give rise to breast neoplasms. The neoplasms are further graded on the basis of their level of anaplasia (gradfes I to III) to reflect their biologic aggressiveness.

Fibroadenoma is characterized as a discrete, encapsulated, solitary free mobile nodules ranging from 1 to 10 cm in diameter. There is loose fibroblastic stroma (currently felt to be the neoplastic component of these benign tumors) containing epithelial lined spaces of that take on a slit like appearance. Phyllodes tumors are similarly sharply demarcated mass of the breast that tend to be larger than fibroadenomas and are characterized by leaflike clefts and a hypercellular stroma with nuclear atypia.

Breast carcinomas arise in the ductal epithelium (90%) or from the terminal duct-lobolar unit (10%). These tumors frequently present as hard fixed palpable lesions that may cause retraction of the overlying skin or a bloody nipple discharge. Both ductal and lobular carcinomas have an in situ form (non-invasive) that is contained in within the confines of the basement membrane and an invasive or infiltrating growth pattern. Both the in situ and the infiltrating carcinomas may present with palpable masses or mammographic abnormalities sush as microcalcifications. The infiltrating lesion frequently is associated with a dense fibrous response so called tumor desmoplasia. Ductal carcinoma is composed of round to polygonal cells that may form well defined glands or sheets of poorly differentiated cells with marked nuclear atypia and high mitotic counts. Invasive lobular carcinoma tend to grow as relatively bland cells growing in sigle rows as cord of cells. They often surround preexisting ducts in a targetoid fashion. Currently breast carcinomas are graded based on three criteria. Tubule or gland formatin, nuclear pleomorphism and mitotic count. A composite of theses three characteristics is used to grade the tumors as either well, moderately or poorly differentiated These grades along with other factors such as tumor size, lymph node status and the presence of estrogen receptors are useful prognostic indicators. All infiltrating carcinomas have the potential to metastasize where as the in situ variety because they are confined by the basement membrane cannot gain access to the vascular lymphatic system.

Nonmetastasizing neoplasms included intraductal (in situ) or in situ lobular. Uncommonly metastasizing neoplasms (low grade) included colloidal, medullary and papillary carcinoma where colloidal and medullary forms represent soft fleshy or bulky patterns of growth. Colloidal form produces mucinous secretions intra and extracellularly. Moderately and poorly differentiated infiltrating ductal and lobular carcinoma included both of low and high grade samples.

Analyses of Breast Tissues

Snap frozen tissue was quickly minced and a portion (0.1 -0.2 g) used for the extraction of enzymes and inhibitors and remainder of the tissue for RNA extraction. Corresponding to each frozen sample, an appropriate and carefully screened paraffin-fixed block (from many) was used to prepare slides for immunohistochemistry to evaluate the presence of MMPs and TIMPs (in different cell types) in the breast tissue.. The snap frozen tissues kept for five years were suitable in some instances for Northerns (mRNA estimation) and most of them for RT - PCR analyses as long as care was taken during the preparation of total tissue RNA. Protein estimation of tissue homogenates were by the use of BioRad protein estimation kit.

Immunohistochemical analysis of breast tissues

Immunohistochemical analysis of breast tissues

Immunohistochemical evaluations were carried out using Histostain^{TM} SP Kit (CAT. # 95-9743) using AEC (aminoethyl carbazole, red signal) or DAB (brown signal) that was purchased from Zymed Laboratories Inc.(Zymed Laboratories, San Francisco, CA) and following the manufacturer's instructions with minor modifications. Paraffin sections (4 microns) were cleared of paraffin, blocked for endogenous peroxidase, washed in water, PBS, blocked with normal horse serum and then were treated with drops of specific primary antibodies in a humidity chamber (1h). The tissues were washed, and treated with a biotinylated secondary antibodies (1-3h), followed by avidin-biotin-peroxidase complex [20]. They were then washed and treated with the chromogen. The slides were counterstained with hematoxylin, washed, dehydrated and evaluated for the localization of various MMPs and TIMPs in specific type of cells or stroma in the tissue. The kit incorporated HorseRadish Peroxidase (HRP), streptavidin, and affinity-purified antibodies into the Labeled-[strept]Avidin-Biotin (LAB-SA) method. The chromogen/substrate system used was [aminoethyl carbazole (AEC) for a red signal or diaminobenzidine (DAB) for a brown signal] that created an intense color deposit around the antigen/antibody/enzyme complex in the tissue. Most of the tissue sections were stained with purified anti-MMPs or -TIMPs. For each breast tissue, additional slides containing no primary IgGs or stained with hematoxylin-eosin stain alone (for cell structures and types) were also included.

Each tissue section was photographed using the microscope and the photographic slides were carefully evaluated with Dr. Scott Sittler to confirm the integrity of the tissue and the structures relating to normal, benign or carcinoma of breast tissues (from hematoxylin-eosin stain slide). The intensity of the color deposit around the antigen/antibody/enzyme complex for each slide were also scored and recorded using grading system of 1-4 with + sign against each number as 1^+, 2^+ to 4. The terminology of negative or positive to IgGs were also used besides the grading system.

Extraction and assays of Matrix metalloproteinases and their inhibitors

All steps were carried out at about 4^{o}C. Human breast tissues were weighed (100-200 mg), minced and homogenized in a laminar flow hood by use of a Polytron (Brinkmann, Westbury, NY) in 7.5 volumes extraction buffer (0.25% Triton X-100 or 1 mol/L guanidine hydrochloride or 0.5 - 2% SDS in 50 mmol/L Tris/HCl buffer, pH 7.5) The homogenates were centrifuged and the pellet re-extracted with 2.5 volumes of appropriate extraction buffer, centrifuged and supernatants combined [21]. Extracts (except SDS) were dialyzed and stored in aliquots at -70^{o}C.

Most tissues contain metalloproteinase inhibitors, TIMP(s). When enzymes were assayed directly in extracts, it was necessary to destroy inhibitors by reduction and alkylation with 2mmol/L dithiothreitol for 30 minutes followed by alkylation with 5 mmol/L iodoacetamide for the same period. Treated samples were then dialyzed against assay buffer (0.05 mol/L Tris-HCl, pH 7.5, 0.1 mol/L NaCl, 0.01 mol/L CaCl2,0.02% NaN3, and 0.005% Brij 35). The inhibitors are destroyed without significantly affecting the proteases [21,22].

Tissue extracts (with or without reduction and alkylation) were assayed and quantitated for various MMPs using ^{3}H-acetylated Type I gelatin to estimate the combined activities of gelatinase A or B [21]; ^{3}H-acetylated telopeptide-free Type I rat skin collagen for MMP-1 (interstitial collagenase) [21] and ^{3}H-carboxymethylated transferrin [23] or ^{3}H-acetylated proteoglycan monomer bead assay [22] for stromelysin (MMP-3) and MMP-7. Blanks were set

up with 1,10-phenanthroline and p-aminophenylmercuric acetate (APMA) used to activate latent enzymes.

Zymography and Immunoprecipitation Analysis.

Gelatin zymography of breast extracts was by the method of Herron et. al.[24] This method detects pg quantities of gelatinase A and B and nanogram quantities of other proteases. (SDS)-polyacrylamide gel electrophoresis [25] was performed in 7.5 or 11% polyacrylamide containing 0.33 mg/ml gelatin under nonreducing conditions. The gels were rinsed twice in 2.5% Triton X-100 for 20 min, at 21° C, to remove SDS and then incubated for 18 hours at 37° C in assay buffer. 3 mmol/L phenylmethylsulfonyl fluoride (PMSF) was included in the Triton wash and the assay buffer to inhibit the digestion of gelatin by serine proteases for all the gels. Gels were stained with Coomassie Brilliant Blue R-250 (0.1% in 45% methyl alcohol for 30 min and destained in 7% acetic acid) Both latent and active forms of gelatinases or other proteases produce clear areas in the gel because latent enzymes become active upon refolding from SDS. Gelatinase A (72 kDa) and gelatinase B (92 kDa) are clearly resolved on the basis of their M_r. The relative amounts of enzymes were quantitated using the imager (Ultra Violet Products (UVP), Upland, CA) and GelBase/GelBlot Pro Software to scan and quantitate the appropriate enzyme bands. Calibrated known amounts of gelatinases and other metalloproteinases were included in each gel containing breast tissue extracts. Units of enzyme activity (each separate enzyme activity) were converted to μg of enzyme protein and in turn expressed as microgram of enzyme/g wet weight breast tissue (μg/g wet weight tissue). The values are presented as mean ± SEM (standard error of mean).

Immunoprecipitation analysis was performed according to standard methods [26] to show the M_r of enzymes and their inhibitors found in the breast tissues. Tissue extracts (diluted or concentrated) were immunoprecipitated with rabbit anti-MMP(s)or anti-TIMP(s) IgG(s) using protein A-agarose suspensions. Blanks were prepared with specific IgG alone, preimmune serum with enzyme fraction, and enzyme with protein A gels but no IgG. After the reacted agarose gels were washed, the immune complexes [26] dissolved in sample buffer and analyzed by zymography for specific enzyme activity or for TIMPs by reverse zymography or for proteins by SDS-PAGE. By this method, 90-95% of the antigen present is immunoprecipitated.

Reverse zymography:

A modified method of Heron et al [24] was used to detect the TIMPs. A kit (University Technologies International Inc., Alberta, Canada) was used to perform reverse zymography of tissue extracts. The kit provided standards of TIMP-1, -2 and -3 and the media containing the enzymes. Tissue extract was fractionated by SDS-PAGE [25] electrophoresis using 12,5% acrylamide , 0.75 mg/ml gelatin solution and the supplied media (0.1 ml) containing enzymes. After washing with 2.5% Triton X-100, the gel was incubated in Tris buffer (37° C, and minimum gentle shaking) and stained with Coomassie blue solution to reveal cleared and uncleared area of the gelatin in the gel. Uncleared blue staining areas were revealed only if TIMPs were present. Quantitation of TIMPs by this method was not possible. However,this method resolved the presence of TIMP-1, -2 and -3 on the basis of M_r.

TIMPs were quantitated by immunoassay (EIA) [27] using kits #QIA31-1EA (TIMP-1) and #QIA40-1EA (TIMP-2) purchased from Bio Trak (Oncogene Research Products, Cambridge, MA).

Quantitation of TIMP-1 by Chemilumunescence Western Blot:

Metalloproteinases and inhibitors were further identified by western blotting. After transblotting the components separated by SDS-PAGE, the membranes were reacted with monospecific rabbit antisera generated against various MMPs and TIMP-1 and-2 in our laboratories. Quantitation of TIMP-1 in breast tissue extrats was carried out by chemiluminescent Western blot analysis using HRP-labeled second antibody. Equal quantity of cellular protein from each tissue extracts were electophoresed in a 12% polyacrylamide gel. After eletrophoretic transfer to a nitrocellulos membrane, the blocked (1% milk protein) membrane was stained with primary (rabbit anti-TIMP-1human, 0.5μg/ml or 1:700) antibody. Washed membrane was then stained with HRP-labelled second antibody followed by Western Blot Chemiluminescence reagent (NEL-102, Dupont, Boston MA) and exposed to Dupont Reflection^{TM} autoradiography film for 10-30 seconds.The film was scanned by an imager and the TIMP related bands were quantitated using the GelBase/GelBlot Pro Software (Ultra Violet Products (UVP), Upland, CA) Quantitated TIMP-1 standard green staining of benign tissue slide whereas **Figure 5B** shows heavy green staining with anti-MT1-MMP was included in each gel.

RNA extraction and reverse transcription polymerase chain reaction (RT - PCR analysis).

Tissues aliquots rapidly frozen in liquid nitrogen and stored at -70^{o}C were used. Total cellular RNA was isolated by a single-step acid guanidium thiocyanate-phenol-chloroform RNA extraction method [28] using TRI REAGENT^{R} ((Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol. Five micrograms of total RNA were reverse-transcribed by using a SuperScript^{TM} kit, Cat# 18089-011 (GIBCOB BRL, Gaithersberg, MD) with random primers. To amplify metalloproteinases (MMP-2, MMP-9, MMP-1, MMP-13), and TIMP-1 or TIMP-2, one-tenth of the cDNA pool was subjected to subsequant PCR amplification by adding 2.5 U of *Taq* DNA polymerase (GIBCO, Cat# 18038-042) to 50 pmol of 5'- and 3'-sequence -specific oligonucleotide primers, dNTP (10 mM, Gibco BRL Cat#18427-013) in a buffer containing 10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCL, and 0.1 mg/ml gelatin, pH 8.3, in 100 μl volume. The mixture was overlaid with mineral and then subjected to amplification in a Model 2400 Perkin-Elmer thermal Cycler (Perkin Elmer, Norwalk, CT). A standard program of 30 cycles was used, consisting of denaturing at 94^{o}C for 90s, annealing at 58^{o}C for 90s, and extension at 72^{o}C for 3 min, and a final extension at 72^{o}C for 5 min. Expression of GAPDH, a housekeeping gene, was also analysed as an internal standard [29,30]. The standard method where each MMP(s), TIMP(s) and GAPDH (housekeeping gene) from one breast tissue was performed singly leading to a semi quantitative expression of MMPs.

For more accurate quantitation of gene expression of enzymes and inhibitors in breast tissues, modified standard RT-PCR method included specific primers for both GAPDH and MMP(s) or TIMP(s) in the same RT-PCR reaction tube enabling the normalization of MMPs & TIMPs to GAPDH values for the final gene expression. The conditions of annealing temperature and number of cycles for MMP-9, MMP-2 and TIMP-1 are shown in **TABLE 1**. Briefly, PCR reaction is carried out using MMP or TIMP specific primers for 5 cycles, annealing temperature at 65^{o}C, then addition of GAPDH specific primers and PCR reaction carried further for 35 cycles.

TABLE 1. PCR Reaction - Modification conditions:

Gene	$Mgcl_2$	Specific primers	Cycles	Annealing temp	GAPDH(ng) primers added	Annealing temp	Cycles
MMP-9	1.6 mM	150 ng	5	65° C	130 ng	65° C	35
MMP-2	1.5 mM	160 ng	5	65° C	130 ng	65° C	35
TIMP-1	1.5 mM	150 ng	2	65° C	130 ng	65° C	35

Primers [29] for MMP-9, MMP-2, MMP-3, MMP-1 (collagenase 1), TIMP-1, TIMP-2 and GAPDH[b] were kindly provided by Dr. V.H. Rao, Meyer Rehabilitation Institute and University of Nebraska Medical Center, Omaha, NE. These primers are synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA). Primer pairs for MMP-13 (collagenase-3) and GAPDH[a] were synthesized using a DNA synthesizer (Perkin Elmer model 374 (Applied Biosystems, Foster City, CA). The primer sets listed below produced one PCR product band Amplified PCR products were identified by electrophoresis of 5 - 10 µl sample aliquotes on 1.5% agarose gel stained with ethidium bromide, visualized by UV transillumination and photographed. Semiquantitation of MMPs and TIMPs are performed using densitometry and comparison to GAPDH (internal standard) from the same sample after using an image analysis system (Ultra Violet Products (UVP) and GelBase/GelBlot Pro Software, CA). The PCR procedure ia repeated at least two or three times for each sample. Prime sequences for MMPs, TIMPs and GAPDH are recorded in **Table 2**. Sets of primers are used as described previously [35,36].

Table 2. Primers for RT-PCR analysis

Genes (bp)		Sequence	Product
MMP-2	Antisense	5′-GCA GCC TAG GGA GTC GGA TTT GAT G-3′	480
	Sense	5′-CCA CGT GAC AAG CCC ATG GGG CCC C-3′	
MMP-9	Antisense	5′-GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT-3′	640
	Sense	5′-GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC-3′	
MMP-1	Antisense	5′-TTC CAG GTA TTT CTG GAC TAA GT-3′	185
	Sense	5′-ATT GGA GCA GCA AGA GGC TGG GA-3′	
MMP-3	Antisense	5′-TTC TAG ATA TTT CTG AAC AAG G-3′	155
	Sense	5′-GCA TAG AGA CAA CAT AGA GCT-3′	
MMP-13	Antisense	5′-GAA CAG CTG CAC TTA Y-3′	134
	Sense	5′-TCA TGA CCT CAT CTT C-3′	
GAPDH[a]	Antisense	5′-TGA TTT TGG AGG GAT CTC GC-3′	230
	Sense	5′-ACG CAT TTG GTC GTA TTG GG-3′	
GAPDH[b]	Antisense	5′-GGA TCT CGC TCC TGG AAG ATG GTG ATG GG-3′	237
	Sense	5′-GGT GAA GGT CGG AGT CAA CGG ATT TGG TCG-3′	
TIMP-1	Antisense	5′-GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT-3′	551
	Sense	5′-TGC ACC TGT GTC CCA CCC CAC CCA CAG AGC-3′	
TIMP-2	Antisense	5′-GGA AGC TTT TAT GGG TCC TCG ATG TCG AG-3′	590
	Sense	5′-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3′	
MMP-14	Antisense	5′-CGC TAC GCC ATC CAG GGT CTC AA-3′	496
	Sense	5′-CGG TCA TCA TCG GGC AGC ACA AA-3′	

RESULTS:

Pathology of breast tissues used for this study is documented in the Methods Section. Collectively, the study included over 200 well characterized normal, benign, and various types and grades of breast carcinoma tissue samples. Of those, 6 normals, 30 benign tumors (fibroadenomas), 3 Phyllodes benign tumors, 12 in situ ductal carcinomas, 8 in situ lobular carcinomas, 6 colloidal carcinomas, 10 infiltrating lobular carcinomas, 30 infiltrating ductal carcinomas grade (low grade) and 55 infiltrating ductal carcinomas grade (high grade) breast tissues were evaluated for quantitation of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) by biochemical analyses. Adjacent to frozen tissues, the parrafin-fixed blocks were available for all tissues, however, some paraffin blocks had insufficient tissues to make 10 slides of tissues needed to evaluate MMPs and TIMPs. Reduced number of breast tissues were also analyzed for RT-PCR analysis as some samples showed completely degraded mRNA.

Quantitation and characterization of MMPs and TIMPs in breast tissue.

The breast tissues were extracted first with 0.25% Triton X-100 solution followed by buffered 1 mole/L guanadine hydrochloride. Triton extracts removed soluble proteins from the tissues and extracted only 40-60% of the MMPs. Buffered 1 mole/L guanadine hydrochloride extracted remainder portion of enzymes and a bulk of TIMPs present in the tissues. 0.1% SDS solution was used as the final solution in the extraction procedure. SDS was used mainly to solubilize any TIMP-3 present in the tissues. The tissue extracts were analyzed by zymography and the gels were scanned by an imager and the cleared bands corresponding to each latent and active enzymes (by M_r) were quantitated using Gelblot/Gelbase software. The arbitary units for each band were quantitated using the arbitary values for calibrated and quantitated standard enzyme that was included in each gel. Treating the gels with with 3 mmol/L phenylmethyl sulfonyl fluoride inhibited the serine proteases bands in the gel. The cleared bands in the gels were identified as gelatinases and this was confirmed when a similar gel was treated with both phenylmethyl sulfonyl fluoride and 10 mmol/L of ethylene diamine tetraacetic acid (EDTA). The gel showed no bands (result not shown) and suggested the presence of MMPs as cleared bands in the original gel.

The arbitary values were converted to the weight of protein in nanograms to micrograms and based finally to the gram wet weight of breast tissue. The TIMPs were also recorded as microgram protein/g wet weight breast tissue. MMPs and TIMPs present in the breast tissue extracts were calculated as μg of enzyme or inhibitor/g wet weight tissue.

The zymographical evaluation of breast tissue extracts (Triton and guanidine hydrochloride) are presented in **Figures 1a and 1b. Figure 1a** shows the profile of 0.25% Triton extracts whereas **Figure 1b** shows thw profile of guanidine hydrochloride tissue extracts. The normal breast tissue extracts showed mainly very low amounts of latent MMP-2 in guanidine extracts. Benign extracts showed only the bands corresponding to latent and active form of MMP-2. No evidence of MMP-9 in any form or high M_r bands were observed in the benign tissues. The low and high grade of infiltrating ductal carcinoma breast tissues showed latent and active forms of MMP-2 and MMP-9 and high M_r bands. Tissue extracts were scanned from each individual breast tissue at three different aliquotes that included quantitated and calibrated aliquotes of MMP-9 and MMP-2 in the same gel. After processing the gels, they were scanned using the imager and the softwear as described in Methods section. The quantitative results are presented in **Table 3.**

The gelatinases were quantitated by zymography and the enzyme substrate assay (tritiated gelatin). The zymography gave the individual values for latent and active ezymes for MMP-9

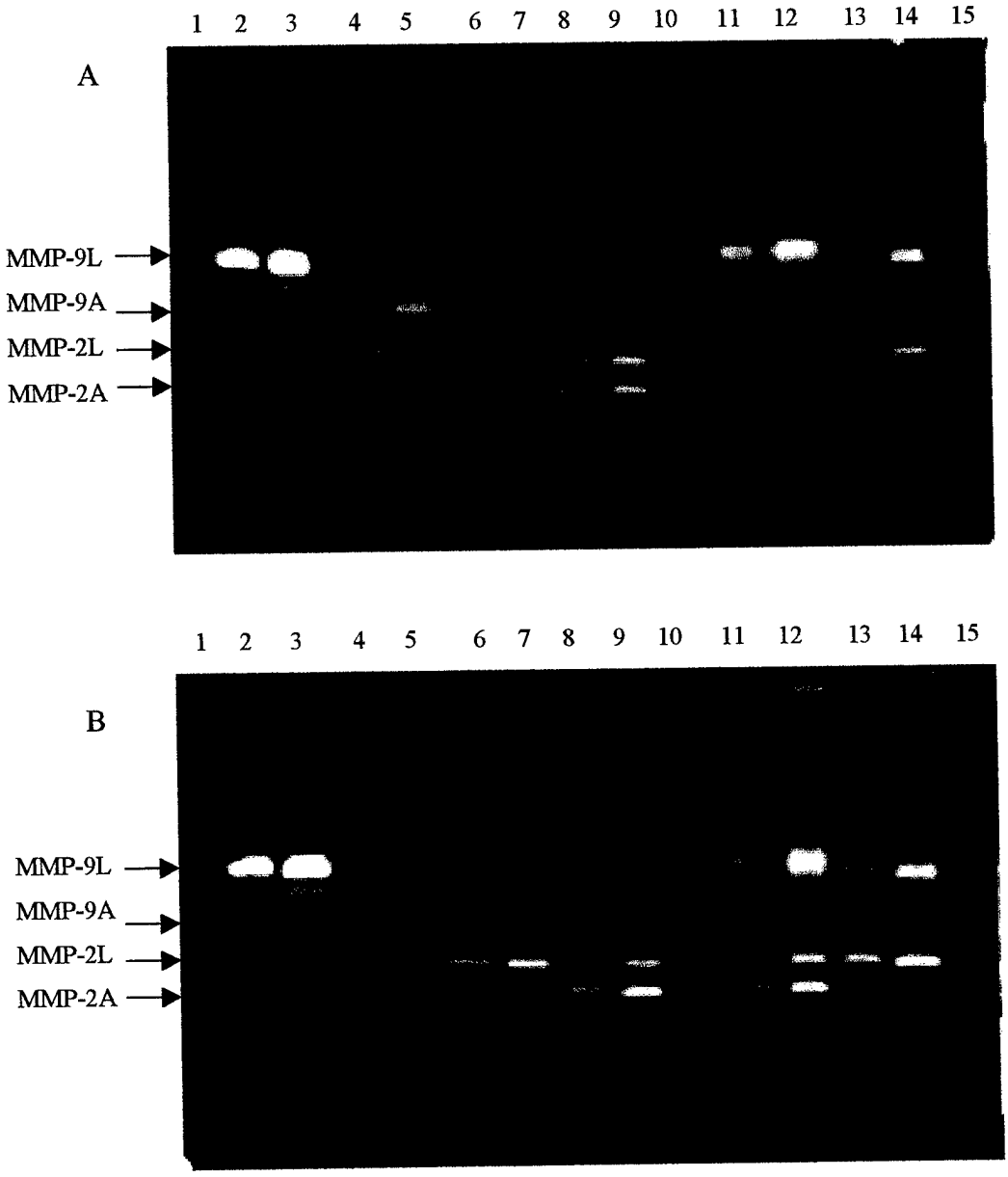


Figure 1 . Gelatin Zymographic Analysis of Normal, Benign , and Carcinoma Breast Tissue Extracts.

Numbers along the top indicate the lanes used. **A:** - shows the analysis of dialyzed 0.25% Triton X-100 breast tissue extracts whereas **B:** - shows the analysis of dialyzed 1mol/L guanidine hydrochloride breast extracts. Zymography was performed as described in Materials and Methods. The extracts were adjusted to similar amounts (1-5μg) per lane. The lanes are the same for each figure **A** and **B**. Lanes 2,3 show latent form of gelatinase B (MMP-9). Lanes 4,5 show active form of MMP-9. Lanes 6 ,7 - two different normal breast tissue extracts showing low amounts of MMPs. Lanes 8,9 - two different benign breast extracts showing only latent and active forms of gelatinase A (MMMP-2). Lanes 10,11 - two different high grade infiltrating ductal carcinoma breast tissues showing latent and active forms of MMP-9, latent and active MMP-2 and other higher M_r bands - dimers of MMP-2 and MMP-9. Lanes 12,13 - showing low grade infiltrating ductal carcinoma breast tissues showing lower amounts of latent and active MMP-9 and high M_r bands than those present in high grade carcinoma. MMP-2 (latent and active) are similar in amount to those found in high grade tissue extracts. Triton extracts contain 35-40% of total enzyme found in the tissue extracts.

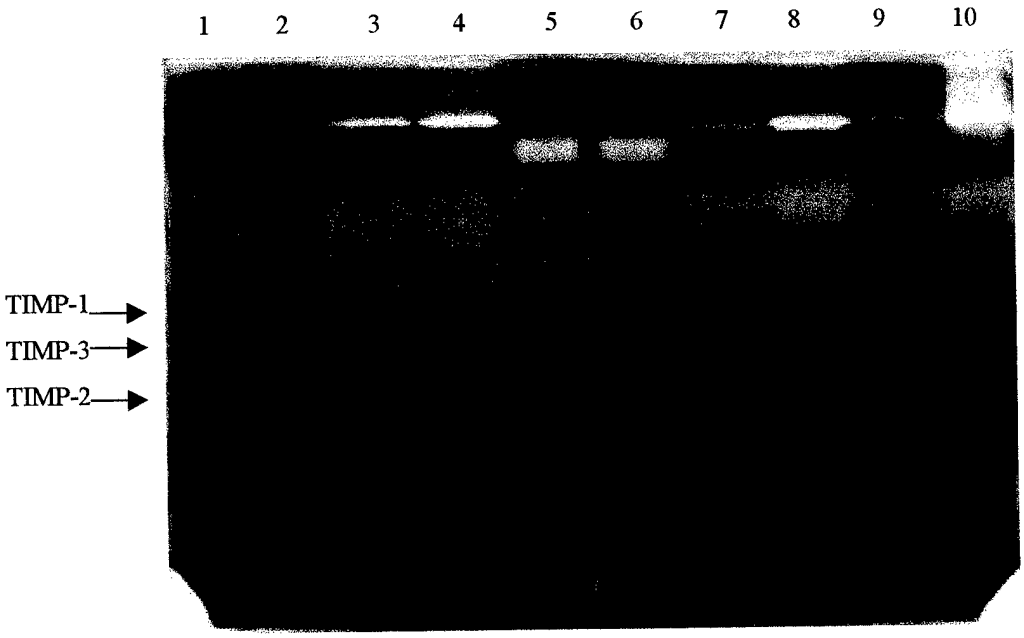


Figure 2. Reverse Zymographical Analysis of Breast Tissue Extracts.

Numbers along the top indicate the lanes used. Aliquots of the dialyzed breast tissue extracts (guanadine hydrochloride) adjusted to 5-10μg were analyzed against TIMP-1, -2 and -3 standards using the kit (UTI Inc. Canada), and 0.75 mg/ml gelatin in 12.5% acrylamide gel. Uncleared blue staining areas were revealed only if TIMPs were present. Lane 1 - Standards TIMP-1, -2 and -3. Lane 2 - M.W. protein standards. Lane s 3,4 - Infiltrating poorly differentiated ductal carcinoma tissue extracts (high grade) showing virtually no TIMP-1 and -2 and low amounts of TIMP-3. Lanes 5,6 - Infiltrating moderately differentiated ductal carcinoma tissue extracts (low grade) showing some TIMP-1 and TIMP-2 and low amounts of TIMP-3. Lanes 7,8 - Benign tissue extracts showing large amounts of TIMP-1 and reasonable amounts of TIMP-2 and -3. . Lane 9 - normal tissue extracts showing TIMP-1, -2, and -3. The TIMPs separated according to M_r could not be quantitated by this method due to the presence of other proteins present in the extracts.

TABLE 3. Quantitation of Matrix Matalloproteinases--Gelatinases (A[@]+B[#]) and Collagenase (MMP-1, or -13)[^] in Human Breast Tissue Extracts[↑].

Tissue	No.	MMP-2[@] + MMP-9[#] μg[*]	MMP-9 L/A	MMP-2 L/A	MMP-9[⊥] μg[*]	MMP-1[^] μg[*]	Total Enzyme μg
Normal	6	0.62 ± 0.02	N/A	7.8	N/A	0.05 ± 0.01	0.67
Benign Tumor	30	1.95 ± 0.03	N/A	4.90	N/A	0.95 ±0.05	2.90
Benign Tumor Phyllodes	3	2.31 ± 0.6	N/A	1.6	N/A	0.45 ± 0.03	2.76
In Situ ductal carcinoma	12	1.50 ± 0.03	1.5	4.30	0.39 ± 0.02	0.18 ± 0.01	2.07
In-Situ lobular carcinoma	8	1.25 ± 0.05	1.2	4.3	0.50 ± 0.03	0.70 ±0.04	2.45
Infiltrating ductal carcinoma high grade	55	9.9 ± 0.10	0.92	1.6	4.8 ± 0.03	1.25 ± 0.02	15.95
Infiltrating ductal carcinoma low grade	30	3.3 ± 0.07	1.85	2.55	0.75 ± 0.02	0.85 ± 0.09	4.90
Infiltrating Lobular carcinoma	10	3.9 ± 0.05	0.82	2.40	1.9 ± 0.01	0.95 ± 0.6	6.75
Colloidal carcinoma	6	1.8 ± 0.04	1.2	2.0	0.95 ± 0.05	0.95 ± 0.03	2.70

Gelatinase A =MMP-2[@] + gelatinase B =MMP-9[#], Total Units = latent + active enzyme from breast extracts.
[↑] 0.25% Triton X-100 extracts + 1 mol/L guanadine hydrochloride extracts.
One enzyme unit = 1μg of substate digested /min at 37° C for gelatinases & at 30° C for collagenase.
[*] Values expressed as μg/g wet weight breast tissue. -- units of enzyme activity converted to μg.
Values are given as mean ± SEM (standard error of the mean).
L/A = Latent enzyme/Active enzyme ratio.
[⊥] Amount of MMP-9 enzyme from total gelatinase - calculated from zymography - imager analysis.
[^] Interstitial collagenase - MMP-1 or MMP-13 or both.
Pathology of breast tissues is reported in the Materials and Methods section.

and MMP-2. The combined values were obtained by substrate assays and these were proportionately equivalent to those obtained by zymography (80%). The interstitial collagenase (MMP-1, collagnase-1) and collagenase-3 (MMP-13) was assayed using tritiated collagen after reduction and alkylation of dialyzed tissue extracts to destroy tissue inhibitors [21]. Both late and active enzymes can be estimated by this method (see Methods and Materials). The results presented in **Table 3**. MMP-1 in many instances cannot be detected by gelatin zymography due to poor digestion of gelatin by MMP-1. Stromelysin-1 or MMP-7 (matrilysin, PUMP-1) were not detected in any of the breast tissue extracts. No bands were obtained in transferrin or casein zymography or detected using enzyme substrate assays with tritiated transferrin. MT1-MMP (membrane type matrix metalloproteinase) was not quantifiable by zymography. Confocal analysis using anti-MT1-MMP) IgGs (kindly provided by Dr. G. Goldberg, Washington university, St. Louis), showed that MT1-MMP was seen only in breast cancer tissues.

Biochemical quantification of MMPs showed an overall increase in all types of MMPs in cancer tissues (**Table 3**). The results in Table 3 indicate that **MMP-9** or gelatinase B was the **key MMP** that had significantly changed from virtually in zero amounts in benign tissue to **4.8** μg in breast carcinoma. MMP-9 was also present at levels **0.39** μg (19.5 units)/g wet weight tissue in *in situ* ductal to **4.8** μg (240 units)/g wet weight tissue in infiltrating ductal carcinoma (high grade). **Lobular** carcinoma also showed **1.9** μg of **MMP-9**. Zymography also shows that a fraction of MMP-9 and MMP-2 are in their active forms. There is a corelation of constitutively expressed MMP-1 or MMP-13 showing **0.05 μg in normal tissues** compared to **1.25** μg/g wet weight tissue) in cancer tissues. Constitutively expressed **MMP-2** also increases from o.62 μg to 5.1 μg in high grade cancer tissues.

The findings in **Tables 3 and 4** categorically show that there is an **imbalance** in which the destructive proteases (**MMPs**) greatly outweigh the controlling inhibitors (**TIMPs**), destroying the basement membrane constraints and thus facilitating the spread of cancer.

It was difficult to find in literature, the analysis of benign breast tissues by zymography. The levels of **MMP-9** are highest in infiltrating and poorly differentiated ductal carcinoma (high grade) breast tissue extracts than any of the other carcinomas . Results in **Table 3** clearly show that **MMP-9, 92 kDa type IV collagenase or gelatinase B** is found in varying amounts in cancer tissues when compared with normal and benign breast tissues.

The breast tissue extracts were scanned for **TIMPs** by reverse zymography. **TIMP-1, -2 & -3** are detected in ng quantities. The tissue extracts are fractionated by SDS-PAGE (12,5% acrylamide, gelatin and enzyme media) electophoresis The uncleared blue bands are due to the TIMPs present in the extracts or conditioned media. All 3 TIMPs are present in different amounts in the breast tissue extracts Of the three different extracts, only 1M GuHCl and SDS extracts can be scanned for TIMPs. The SDS extraction step is necessary to remove TIMP-3 entrapped in the matrix of the tissue. The Triton extracts contain other soluble proteins and it becomes difficult to distinguish the TIMP bands from the regular same molecular weight protein bands that did not diffuse out of gel during incubation. Conditioned cell media can be successfully quantitated for TIMPs. Protein profiles by SDS-PAGE electrophoresis were obtained parallel with reverse zymography of Triton and 1 M guanadine extracts. the rational of scanning both gels i.e. reverse zymography and protein gels to remove the density of protein bands to give us the amounts TIMPs present in each breast tissue. This was a futile attempt. The smaal amounts of TIMPs present in the tissues is overwhelmed by larger proportion of the same molecular weight proteins.

Western blot method was next cosidered to quantitate the reacted TIMP bands with rabbit **anti-TIMP-1 & -2** using sensitive chemiluminesce method (see Methods section for details).

Table 4. Amounts of Tissue Inhibitors of Metalloproteinases (TIMPs -1, -2) in dialyzed Triton X-100 & 1M GuHCl extracts of Breast Tissues.

Tissue	No.	TIMP-1 μg*	TIMP-2 μg*	Total
Normal	6	3.9	1..9	5.8
Benign	15	2.1 ± 0.04	3.5 ± 0.03	5.6
infiltrating ductal carcinoma High grade	32	0.15 ± 0.05	1.55 ± 0.15	1.7
Low grade	18	1.0 ± 0.05	2.50 ± 0.04	3.5
In situ ductal carcinoma	5	1.95 ± 0.12	2.9 ± 0.09	4.85

*Breast tissue extracts (Triton and GuHCl) are analyzed for TIMP-1 and TIMP-2 (μg/g wet weight tissue) by TIMP-1 (Cat # QIA31) & TIMP-2 (CAT#QIA40) ELISA kits (BIOTRAK) purchased from Calbiochem (Oncogene Research Products).
For TIMP-1, the diluted extracts showed different values to those obtained for undiluted extracts and these values were higher than those obtained from reverse zymographical-imager analysis. Caliberated TIMP standards of known values (ng) are used.
Infiltrating ductal Carcinoma - high grade or low grade

This method is frequently used in quantitating TIMPs in cell conditioned media. Dialyzed breast tissue extracts were eletrophoretically separated transfered to nitrocellulose paper. The paper was stained with primary and secondary antibodies and TIMP bands to be revealed on X-ray film. This method was not as sensitive as reverse zymography or ELISA kits. A faint band could be seen with 60 ng of TIMP-1. Other protein bands at higher molecular weight also reacted showing smear upto just above TIMP-1 band (result not shown).

The dialyzed breast tissue extracts were quantitated for TIMP-1 and TIMP-2 by ELISA systems purchased from Oncogene Research Products (Calbiochem, Cambridge, MA). This procedure was not satisfactory to quantitate absolute amounts of TIMPs present in the tissue. Solutions containing TIMPs standards showed a good corelation upon dilution of the solutions. However, this was not the case with tissue extracts. Dilutions of breast extract solutions did not match with undiluted dialyzed breast tissue extracts. So we analyzed some of the tissue extracts (undiluted) by ELISA and results are reported in **Table 4**. This Table shows (although may be inaccurate in absolute amounts) however, a trend of lower amounts of TIMP-1 and TIMP-2 (μg/g wet weight tissue) found in **55** cancer tissues compared to the higher amount found in normal (**6** samples) and benign (**15** samples) tissue.

The reults shown in **Tables 3 and 4** clearly indicate that the amounts of MMPs present in cancer breast tissues (10 μg, Grade III cancer) has available only **1.5 -3.5μg** of **TIMPs** for arresting the destruction of tissue by MMPs. There are other MMPs and TIMPs present in the tissue and their quantitation has not been accounted in this study..

Immunohistochemical evaluations of breast tissue:

Immunohistochemical analyses of over 100 breast tissue samples for various MMPs and TIMPs were carried out. Over 1000 slides (10 slides per each breast tissue) were processed. The purified IgGs were used for each MMPs and TIMPs. MT1-MMP was evaluated by confocal analysis. The slides that best represent each type of breast cancer were used.

Each tissue section was photographed for a photographic slide using the microscope available in the Pathology Department, University of Miami. The slides are carefully evaluated with Dr. Scott Sittler, and recorded using grading system of 1-4 with + sign against each number as 1^+, 2^+ to 4. The terminology of negative or positive to IgGs are also used besides the grading system. All IgGs used in this study were monospecific and evaluated priodically by western blots.

Eleven types of breast tissues showing normal, benign and vaious types of carcinomas were stained for various MMPs and TIMPs. The staining of normal breast tissue showed virtually very little staining with anti-MMP-9 and shows staining only with MMP-2, MMP-1, TIMP-1 and TIMP-2. No staining was observed with MMP-3 or MMP-7. Staining of benign, fibroadenoma breast tissue sections, **Figure 3** revealed virtually no staining with anti-MMP-9 IgGs , but stains with anti-MMP-2. Anti-MMP-1 IgGs revealed staining of stroma (**Figure 3f**) only. The slides representing **Infiltrating and poorly differentiated ductal carcinoma, (IDC, high grade**) breast tissues are presented in **Figure 4**. Anti-MMP-1 (**Figure 4c**) shows staining of 3^+ mainly in tumor cells. Staining with **anti-MMP-9 IgGs** showed 3^+ staining of tumor cells only. Figure **4f and 4g** show that the staining is exclusively in the cytoplasm of tumor cells and no staining of stroma around tumor cells. The staining in the cytoplsm is clear cut and this type of staining was observed in fifty breast tissues representing the high grade infiltrating carcinoma. Three similarly characterized breast tissues showed faint red staining of tumor cells. Anti-MMP-2 IgGs showed staining 2^+ of stroma (**Figure 4h, k, and m**) and no staining was observed in tumor cells. Only weak staining of 1^+ was observed with anti-TIMP-1 (**Figure 4d**). The observation of high staining of MMP-1 and MMP-9 and low staining of TIMP-1 **suggests**

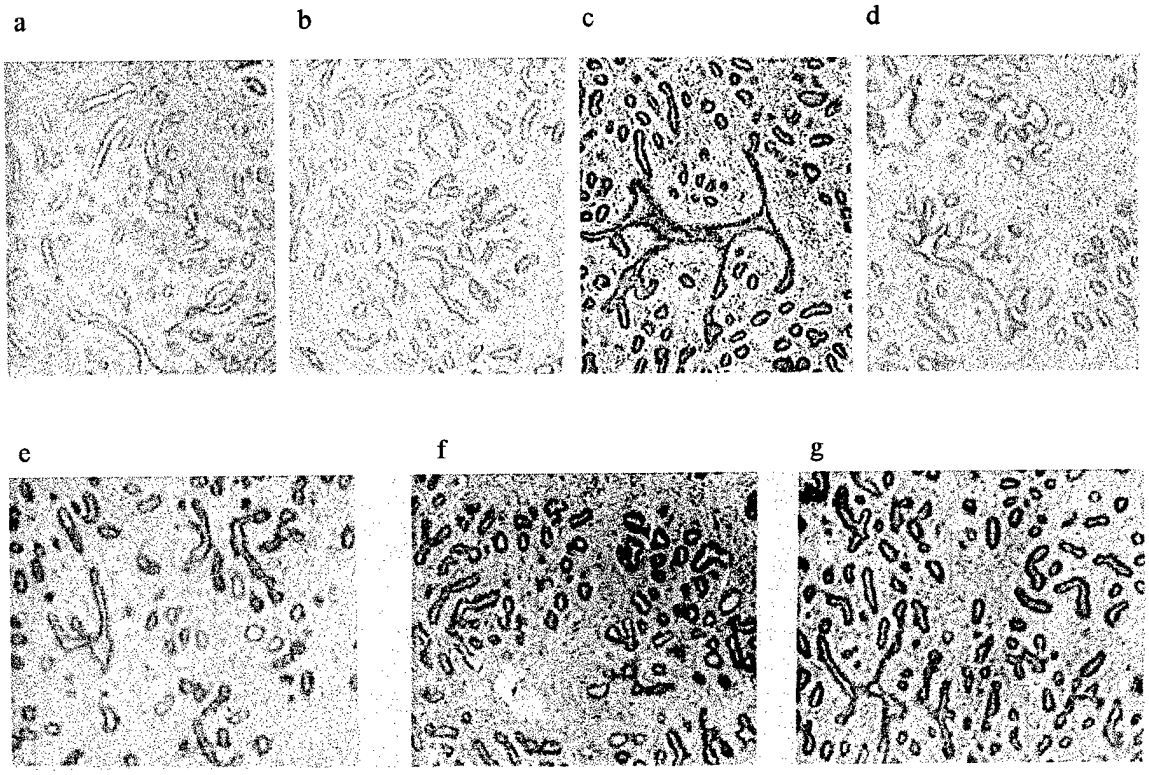


Figure 3: Immunohistochemical staining of human breast tissue **(benign tumor, fibroadenoma)** using immunoperoxidase reagents revealing location of the antigen as **red deposit** with aminoethyl carbazole (AEC) substrate-chromogen. **a: H&E stain. b: no primary IgGs. c: anti-MMP-**9 showing very weak staining of epithelium and stroma. **d: anti-MMP-1** showing 1^+ staining of stroma and weak staining of epithelium. e: **anti-MMP-2** showing 3^+ staining of epithelium and weak staining of stroma. f: **anti-TIMP-1** showing 1^+ of stroma only. g: **anti-TIMP-2** showing 1^+ staining of both epithelium and stroma. (x10)

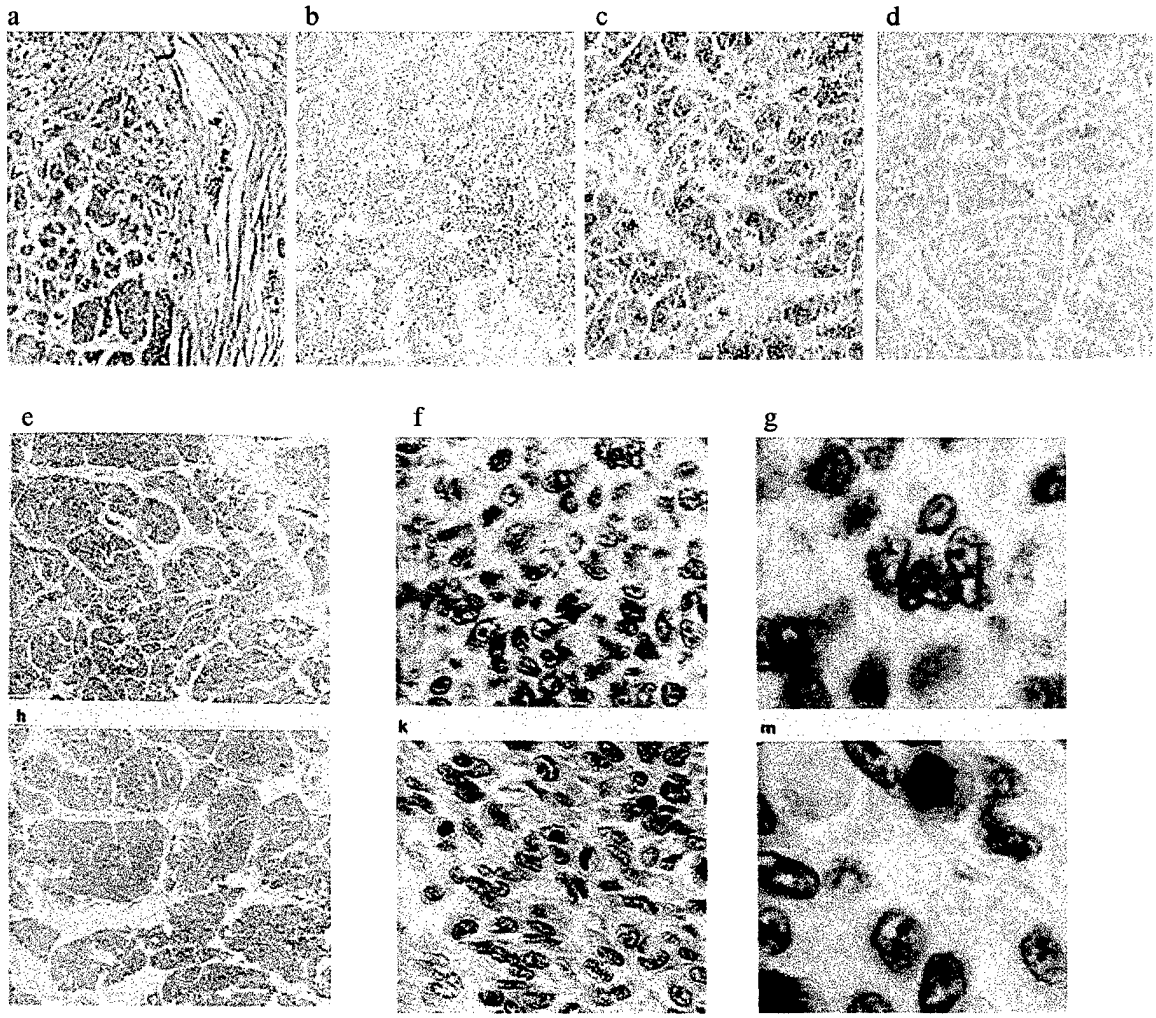


Figure 4: Immunohistochemical staining of human breast tissue **(infiltrating and poorly differentiated ductal [IDC] carcinoma, high grade)** using immunoperoxidase reagents revealing location of the antigen as red deposit with aminoethyl carbazole (AEC) substrate-chromogen. **a: H&E stain. b: no primary IgGs. c: anti-MMP-1** showing 3^+ staining of mainly tumor cells only. **d: anti-TIMP-1** showing 1^+ staining of stroma and some staining of tumor cells. **e: anti-MMP-9 [x10]. f: anti-MMP-9 [x40]. g: anti-MMP-9 [x100]** showing 3^+ staining of tumor cells only. **h: anti-MMP-2 [x10]. k: anti-MMP-2 [x40]. m: anti-MMP-2 [x100].**

an imbalance between MMPs and TIMPs in IDC high grade infiltrating ductal carcinoma breast tissues.

Figure 5 shows immunofluorescent staining of **membrane type 1-MMP** using confocal microscope. The tissue slides were reacted with rabbit anti-MT1-MMP (kindly provided be Dr. G. Goldberg, St Louis) followed by fluorescein-labeled (green color) goat anti rabbit IgG. The staining was scanned by confocal microscope and staining of tissue photographed. **Figure 5A** shows virtually no staining in benign breast tissue section. with anti-MT1-MMP IgGs. **Figure 5B** shows heavy staining with anti-MT1-MMP IgGs using poorly differentiated infiltrating carcinoma tissue section. In all 10 different tissue slides for benign and high grade carcinoma breast tissues were evaluated. Presence of elevated MT1-MMP would further activate any latent MMP-2 present in the tissues. This suggests that yet another MMP together with MMP-9, MMP-1 and MMP-2 present in high amounts in high grade breast tissues overcome the low amounts of TIMPs (Table 4) and degrade the ECM..

Figure 6a,b,c,d show staining of human breast tissue (lobular carcinoma, in situ). **a:** anti-MMP-9 showing 3^+ staining equally of epithelium and stroma. **b:** anti-MMP-2 showing 2^+ staining of tumor cells and surrounding stroma. **c:** high intense staining of stroma and epithelium with anti-MMP-1. **d:** anti-TIMP-1 showing low staining of stroma and epithelium. **Figure 7.** shows staining of colloidal carcinoma tissue. **a:** anti-MMP-1 shows 3^+ staining of tumor cells and stroma. **b:** shows the 3^+ staining of tumor cells and weak staining of stroma. **c:** anti-MMP-2 and **d:** anti TIMP-1. Immunohistochemical analyses categorically show that TIMP(s) staining is weak in carcinoma tissues while they stain highly with gelatinases and MMP-1. No specific staining was observed with anti-MMP-3 or anti-MMP-7 IgGs in breast carcinoma tissues.

Reverse transcription polymerase chain reaction (RT-PCR) analysis.

Detection of low-abundance mRNAs by RT-PCR has now become a standard technique to determine gene expression in tissues. To determine relative or absolute copy numbers of specific mRNAs is difficult without internal standards used as controls to circumvent the sample to sample variation. We examined and quantitated the expression of MMPs and TIMPs in breast tissue samples using RT-PCR analyses. Northern blot analysis were not successful using RNA isolated from various breast tissues. The RNA was in some instances partially degraded. Freshly harvested breast tissue and cells from different cell lines showed the expression of MMPs and TIMPs by northern blots. The feasibility of acquiring 100 plus fresh samples for this multipronged study was impossible. Purified specific primer pairs were kindly provided by Dr. V.H. Rao, Veterans Hospital, . The primers selected for amplification (see **Table 2**) have been shown to produce a single product of the appropriate size for MMP-9, MMP-2 [33], TIMP-2, TIMP-1 and GAPDH, a house keeping gene [33]. We were successful in processing breast tissue samples.

The results obtained by the standard RT-PCR method were semi quantitative and the results were reported in 1997 report. There seemed to be a variation in the expression of GAPDH. Relationship between GAPDH and MMPs and TIMPs remianed questionable.

Modification of standard RT-PCR method was necessary to include GAPDH primers in each MMP and TIMP primers in the same reaction tube (see Methods section). This modification would allow the normalization of MMPs and TIMPs to GAPDH expression in each tissue. We processed over **50** breast tissue samples containing benign and various carcinoma samples. The representitave results reported in **Figures 8 & 9**.

RT-PCR method proved to be useful and at least could utilize the extracted RNA's from one to five year frozen breast tissue samples that we have processed for zymography and

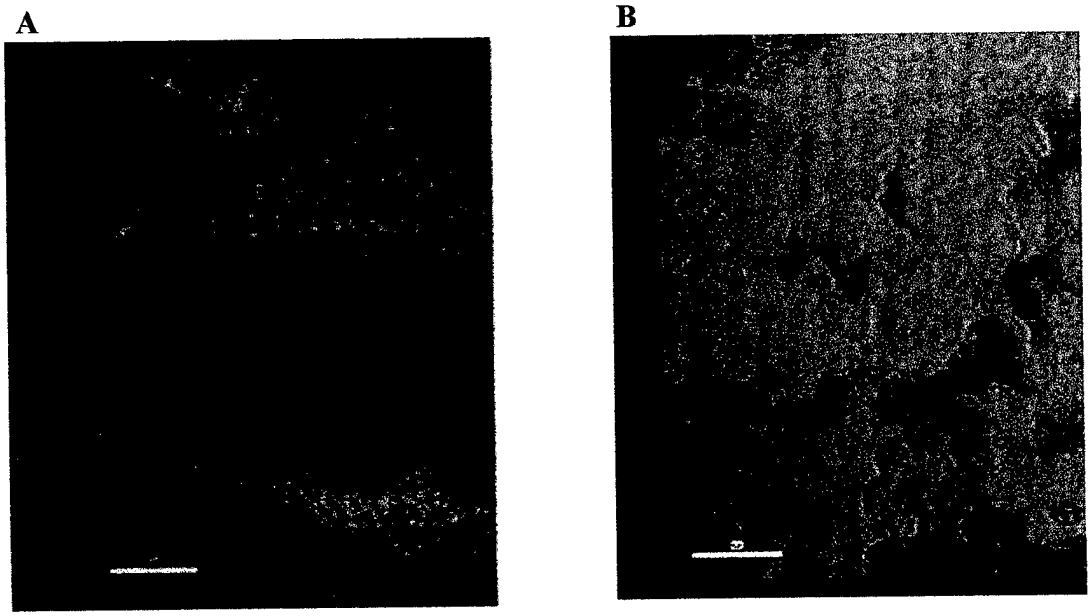


Figure 5: immunofluorescence staining of Membrane type 1-matrix metalloproteinase **(MT-1MMP)** in breast tissues using **confocal microscope.** Rabbit anti-MT1-MMP (kindly provided by Dr. G. Goldberg, St. Louis) was used followed by fluorescein-labeled (green color) goat anti-rabbit IgG.
A: Benign tissue **B: Poorly differentiated infiltrating ductal carcinoma, high grade.**

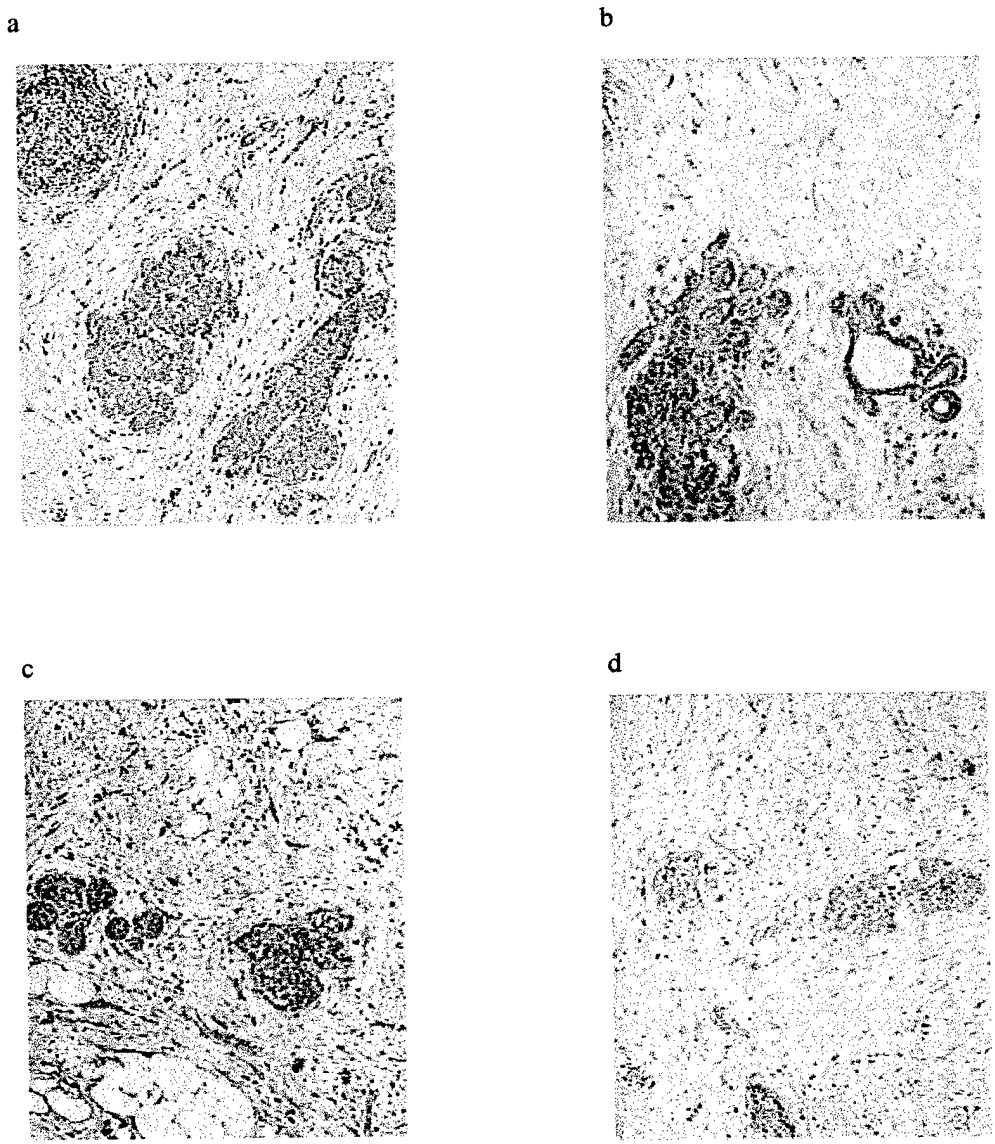


Figure 6: Immunohistochemical staining of human breast tissue (**lobular carcinoma, *in situ***) using immunoperoxidase reagents revealing location of the antigen as **red deposit** with aminoethyl carbazole (**AEC**) substrate-chromogen. **a: anti-MMP-9** showing 3^+ staining equally of epithelium and stroma. **b: anti-MMP-2** showing 2^+ staining of tumor cells and surrounding stroma. **c: anti-MMP-1** showing 4^+ staining equally epithelium and stroma. **d: anti-TIMP-1** showing 1^+ staining of stroma and epithelium. (x10).

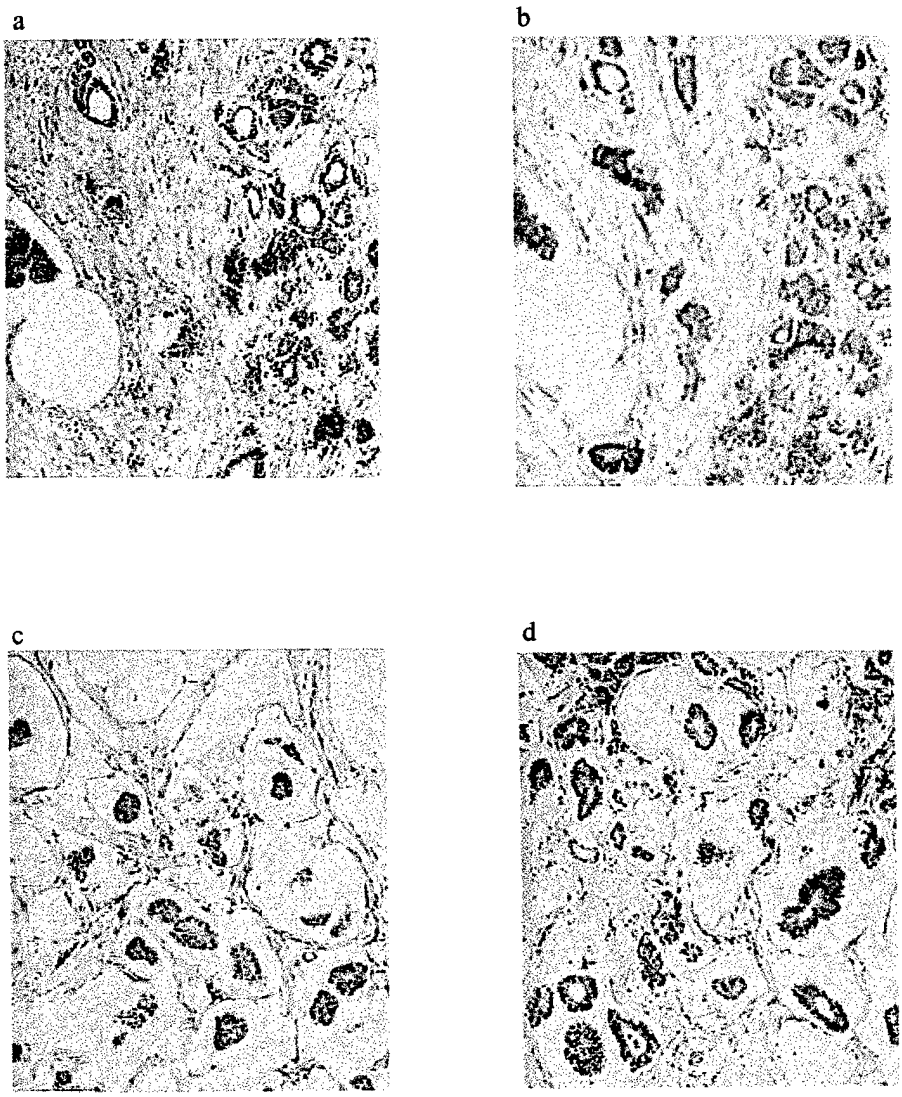
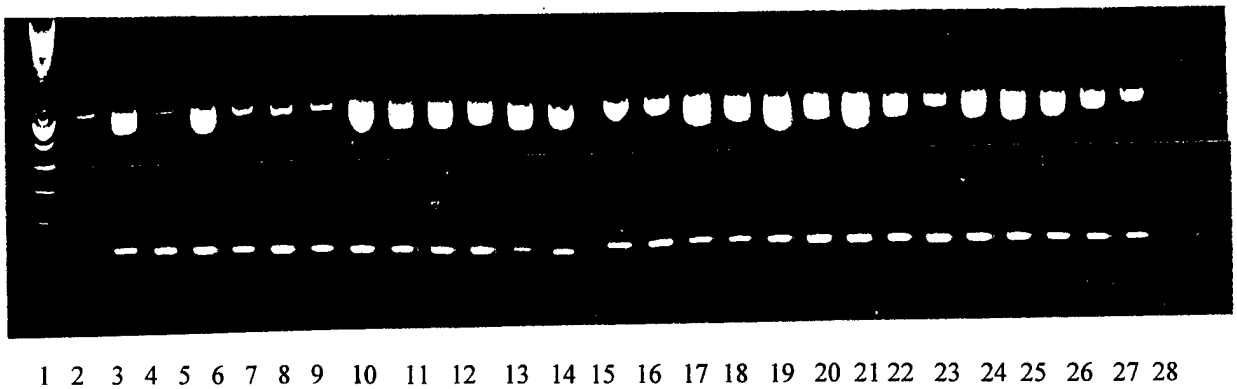


Figure 7: Immunohistochemical staining of human breast tissue **(colloidal carcinoma)** using immunoperoxidase reagents revealing location of the antigen as **red deposit** with aminoethyl carbazole (**AEC**) substrate-chromogen. **a: anti-MMP-1** showing 3^+ staining equally of tumor cells and stroma. **b: anti-MMP-9** showing 3^+ staining of tumor cells and weaker staining of stroma. **c: anti-MMP-2** showing 1^+ staining of tumor cells only. **d: anti-TIMP-1** showing weak staining of stroma. (x10).

A:



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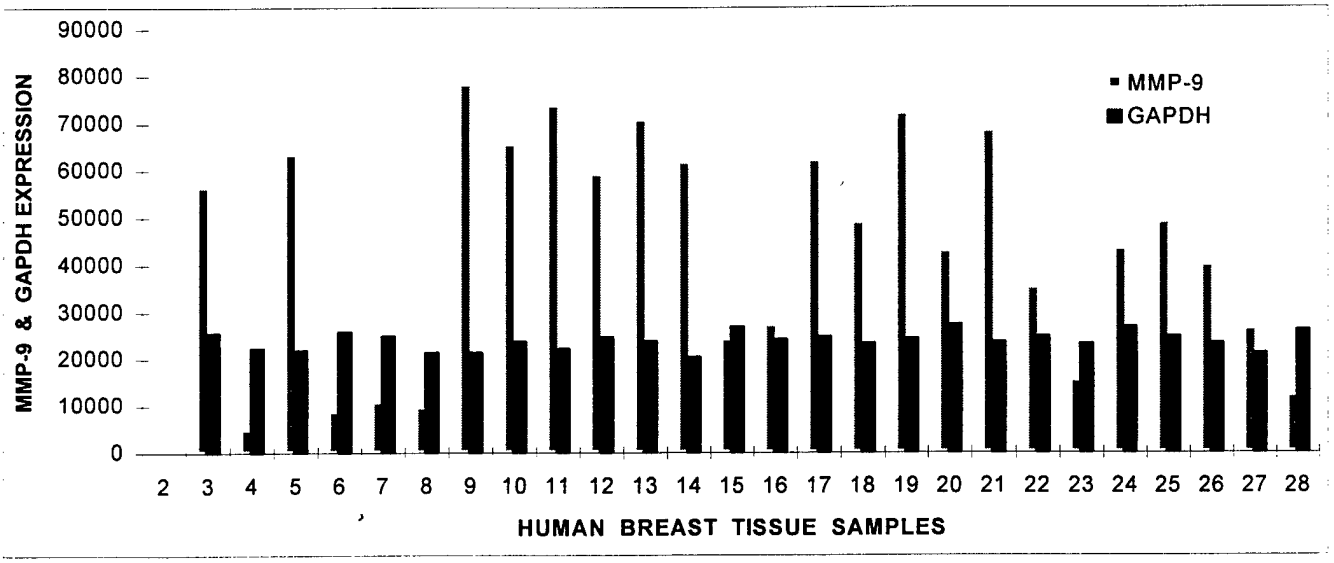

Figure 8. (a) Expression of **MMP-9** (**640** bp fragment) and **GAPDH** (**230** bp fragment) as determined by **PCR.** CDNA was prepared from breast tissues and **RT-PCR** was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. **Lane 1, DNA marker; lane 2, normal breast tissue; lanes 4, 6 , 7, 8, 28, benign breast tissues; lanes 15, 16, 20, 23, 27, infiltrating ductal carcinoma, Grade II; lanes 3, 5, 9, 10, 11, 12, 13, 14, 17, 19, 21, infiltrating poorly differentiated ductal carcinoma breast tissues; lanes, 18, 22, 24, 25, 26, lobular carcinoma.** (b) Semi-quantitative analysis of **MMP-9** in breast tissues. The **MMP-9** & **GAPDH** bands from the same breast tissue samples (and in the same PCR reaction tube) were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

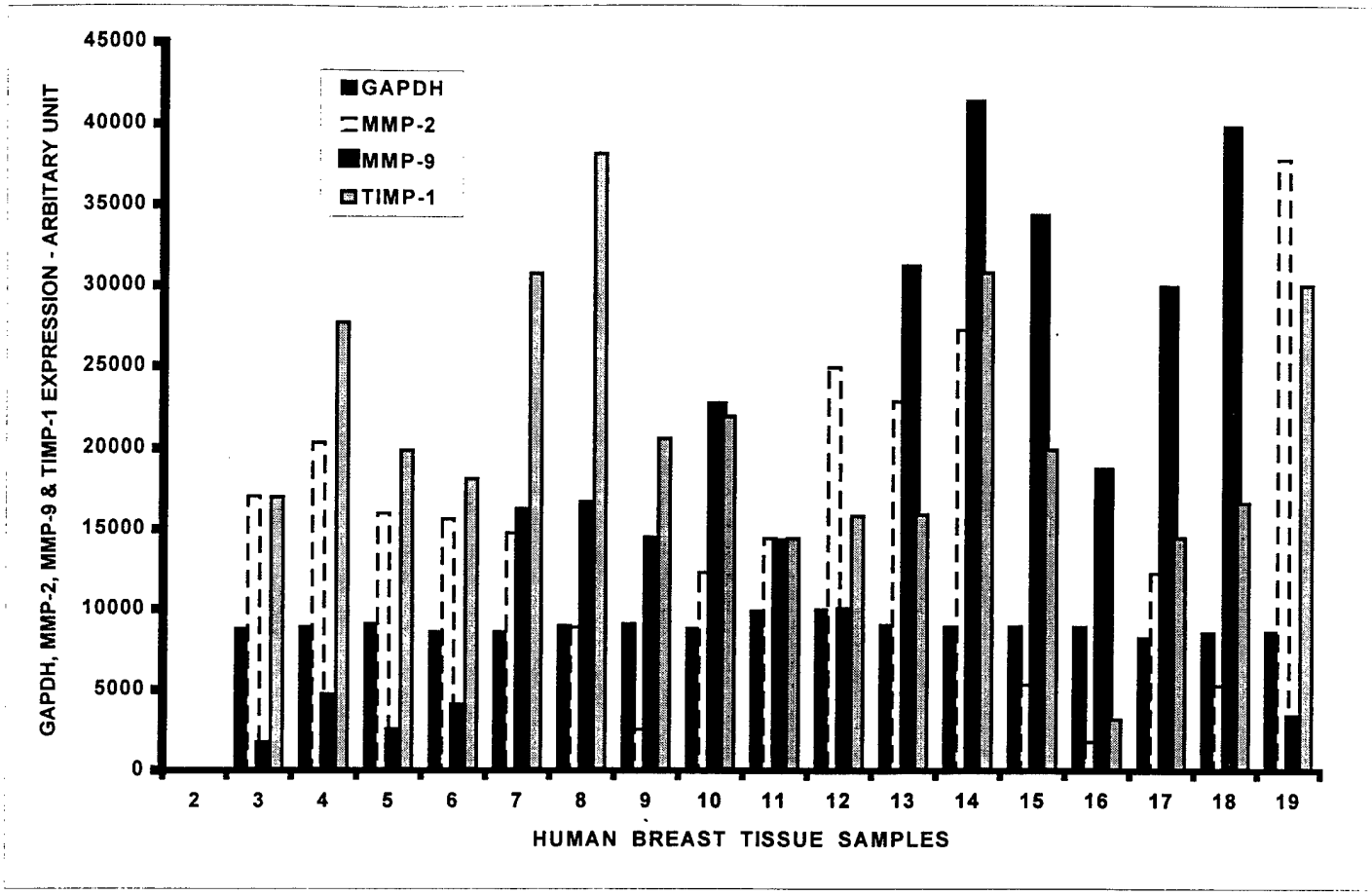

Figure 9. Quantitative Expression of GAPDH, MMP-2, MMP-9 & TIMP-1 in Breast Tissue Samples

Expression of **GAPDH** (**230** bp fragment), **MMP-2** (**480** bp fragment), **MMP-9** (**640** bp fragment) & **TIMP-1** (**551** bp fragment) as determined by PCR. cDNA was prepared from breast tissues and **RT-PCR** was performed using specific primer pairs for each MMP or TIMP-1 & GAPDH in the same reaction tube (details **Table 1**.in the Methods). The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. The bands thus obtained were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products). Units of peak area are arbitaty. However, the values were **normalized** to GAPDH units for MMPs & TIMP-1 in each breast tissue. Lanes **3, 4, 5 & 6** showing **very low** expression of **MMP-9** in **benign** breast tissues; lanes **7 & 8** showing **higher** expression of **MMP-9 & TIMP-1** in ***in situ*** **ductal** carcinoma; lanes **9 & 10** showing **lower** expression of **MMP-2** & **higher** expression of **MMP-9** in ***in situ*** **lobular** carcinoma; lanes **11 & 12** showing higher expression of **MMP-2 & MMP-9** & comparatively **lower** expression of **TIMP-1** in **Grade II, infiltrating ductal** carcinoma; lanes **13, 14 & 15** showing **highest** expression of **MMP-9, MMP-2** & comparatively **lower** expression of **TIMP-1** in **Grade III, infiltrating & poorly differentiated ductal** carcinoma; lanes **16 & 17** showing **lower** expression of **TIMP-1** in **infiltrating lobular carcinoma**; Lane **18** showing **highest** expression of **MMP-9** only **in metaplastic poorly differentiated** carcinoma and lane **19** showing **lower** expression of **MMP-9** in **benign, fibrocystic** breast tissue.

immunohistochemical analysis. Exact number of expressed copies can be acheived by competitive RNA templates for detection of MMPs and TIMPs by RT-PCR procedure described by Tarnuzzer et al. [32]. This method will eliminate sample to sample variation and several MMPs and TIMPs can be expressed from as little as 4 μg of total RNA. This method is time consuming in processing several breast samples.

The results obtained by RT-PCR for MMP-9 and GAPDH expression are reported in **Figure 8.** The figure is self explanatory. Higher amounts of MMP-9 gene expression is evident in high grade infiltrating ductal carcinoma. So far, we have analyzed over **70** breast tissue samples by the **modified RT-PCR method. Figure 9** shows the expression of **GAPDH, MMP-2, MMP-9 and TIMP-1**. The corresponding bands were then analyzed using imager and quantitated by **GELBLOT/GELPRO software** and values are reported in **Figure 9**. Units of peak are arbitary. Normalisation of MMPs and TIMPs expression to GAPDH expression in the same breast tissue eliminates the variation in absolute values. Lanes **3, 4, 5 & 6** showing **very low** expression of **MMP-9** in **benign** breast tissues; lanes **7 & 8** showing **higher** expression of **MMP-9 & TIMP-1** in ***in situ*** **ductal** carcinoma; lanes **9 & 10** showing **lower** expression of **MMP-2** & **higher** expression of **MMP-9** in ***in situ*** **lobular** carcinoma; lanes **11 & 12** showing higher expression of **MMP-2 & MMP-9** & comparatively **lower** expression of **TIMP-**1 in **Grade II, infiltrating ductal** carcinoma; lanes **13, 14 & 15** showing **highest** expression of **MMP-9, MMP-2** & comparatively **lower** expression of **TIMP-1** in **Grade III, infiltrating & poorly differentiated ductal** carcinoma*;* lanes **16 & 17** showing **lower** expression of **TIMP-1** in **infiltrating lobular carcinoma**; Lane **18** showing **highest** expression of **MMP-9** only **in metaplastic poorly differentiated** carcinoma and lane **19** showing **lower** expression of **MMP-9** in **benign, fibrocystic** breast tissue.

The development of the **RT-PCR** procedures provide facile methods for investigating the expression of MMPs and TIMPs.

Using standard RT-PCR procedure and specific primer pairs for **MMP-1 (collagenase -1)** showed a single band in most breast tissues for the expression of **MMP-1 of 185 bp**. With **MMP-13** specific primers, there were two distinct bands suggesting changes in annealing temperature and number of cycles. This part of the project is in progress. Preliminary results show that MMP-13 is mainly expressed in metaplastic and high grade infiltrating ductal carcinoma tissues. MMP-1 is expressed in all breast tissues. MMP-1 expression seems constitutive.

Tissue explant cultures:

breast tissue. Recently five more fresh tissue were obtained, expanded and media from epithelial and fibroblast cell lines have been frozen to be evaluated shortly. This study will also generate a publication that will be most important in un derstanding the secretion of MMP-9 by epithelial tumor cell lines. Himelstein et al [33] found that tumor epithelial cell lines secrete The method of procedure for tissue explant culture was not included in the Materials and Methods section. Fresh (not frozen) biopsied breast tissues consisting of two benign, two high grade infiltrating ductal carcinoma and one *in situ* ductal carcinoma tissues were obtained to evaluate the explant cultures. Procedures used to obtain successful explant culture of epithelial cell line with the prostate tissue explants [31] were followed. The tissue was cut in several small pieces and the media supporting the epithelial was added. The epithelial cell line growth was expanded in 6-well culture dishes with F-12 media containing 10% fetal calf serum for 24 hours. After washing cells with PBS the cells were maintained in serum free media for further 24, 48 and 72 hours. The media were collected, filtered, concentrated and analyzed for MMPs and TIMPs by zymography,and reverse zymography. The epithelial cell line showed only the presence of

MMP-9 by zymography (result not shown) in 72 hour culture.. We also attempted to obtain a fibroblast cells from tissue explant cultures using FGM media. The fibroblastic cells were contaminated with fungi and were discarded. The next two attempts were successful in obtaining both the expanded epithelial and fibroblst cell line. The results by zymography showed the presence of higher amounts of MMP-9 in expanded epithelial cell media than that observed in benign tissue expanded epithelial cell line. No MMP-2 was secreted by the epithelial cell culture. Fibroblast cell culture showed secretion of MMP-2 in media of both benign and carcinoma d MMP-9 only when co-cultured with fibroblast cell line. This was not the case in our findings.

Colocalization of MMP-9 and CD44.

The results from this study genrated a manuscript in collaboration with Dr. Lily Bourguignon, Cell Biology and Anatomy Department, University of Medical School, Florida. The copy of manuscript is included in the **Appendix., Item # 1.**
Refernce: Bourguignon, L.Y., **Gunja-Smith, Z.**, Iida, N., Zhu, H.B., Young, L.J., Muller, W.J. and Cardiff, R.D. **CD44v (3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. Journal of Cellular Physiology. 176: 206-215, 1998.**
This manuscript rlates to the approved statement of Work - in part of Task 3 in SOW

Role of Matrix Metalloproteinases and their Tissue Inhibitors in Human Adenocarcinoma.
Gunja-Smith, Z., Sittler, S.Y., Liu, Y., Woessner, J.F.W.Jr. and Morales, A.R.
The abstract was submitted for the meeting, "An Era of Hope" organized by Department of Defense in Washington DC and held October 31- November 4, 1997. The copy of abstract is included in the **Appendix, Item # 2.** This abstract relates to the preliminary results using smaller number of tissue samples for Task 1, Task 2, and Task 3 in the statement of work.

Expression of MMP-9 in cell lines.
Expression of Gelatinase B (MMP-9) in MET-1 (invasive, mouse) and MCF-7 (human, non-invasice) Breast Cancer Cell Lines. **Z. Gunja-Smith**, Y. Liu and L Bourguignon. Univ. of Miami Medical School, Miami, Fl. Faseb Journal volume 11, 1997. The findings were presented at 17th International Congress of Biochemistry and Molecular Biology San Francisco, CA. The findings are a part of study evaluating the secretion of MMPs and TIMPs in tumor cell lines. The copy of abstract is included in the **Appendix, Item # 3.**

Appendix, Item # 4 represents following article.
Gunja-Smith, Z., Liu, Y. and Sittler, S.Y. Expression of matrix metalloproteinase-9 [Gelatinase B] and a tissue inhibitor of metalloproteinase [TIMP-1] in human breast adenocarcinoma. In Proceedings of the 1998 Miami Nature Biotechnology Winter Symposium, Oxford University Press, Miami Nature Biology Short Reports, 9:115-116. (1998)
This publication represents the Tasks 1,2 and 3 of the approved statement of work. The unified multipronged (biochemical, immunohistochemical and molecular biological techniques) study confirmed that there is an elevated expression of MMP-9 and an underexpression of TIMP-1 in breast carcinoma tissues. This observed balance of MMP-9 over tissue inhibitor (TIMP-1) would lead to an increased matrix degradation and contribute to the invasiveness or metastatic potential of human breast carcinomas.

Rivero, JA, **Gunja-Smith, Z**, Alamino M, Herbert L, Charyulu, VL, Lopez, DM. Signalling events involved in MMP-9 expression in T cells of mammary tumor bearing mice. AACR meeting, Philadelphia, volume 40:pg 471.1999.
Appendix, ITEM # 5

(7) KEY RESEARCH ACCOMPLISHMENTS:

----- Proposed hypothesis -- The small amounts of matrix metalloproteinases (MMPs) produced in normal tissues are held in check by tissue inhibitors (TIMPs), but increased production of MMPs and or decreased production of their inhibitors (TIMPs) have been implicated in tumor invasion and metastasis.

-----The hypothesis was tested using normal, benign and carcinoma (low and high grade potential) breast tissue samples.

-----Over **200 breast samples** were analyzed using the **multipronged study** of **biochemical, immunohistochemical** and **molecular biological techniques** to evaluate the role of matrix metalloproteinases(MMPs) and their tissue inhibitors (TIMPs) in breast cancer using the **same breast tissue** for all the analyses..

-----The breast tissues consisted of **6 normals, 30 benign (fibroadenomas), 3 phyllodes tumor (benign), 12 in situ ductal carcinomas, 8 in situ lobular carcinomas, 6 colloidal carcinoma (non metastasizing potential), 10 infiltrating lobular carcinomas, 30 low grade infiltrating ductal carcinomas** and **55 high grade poorly differentiated and infiltrating ductal carcinomas.**

----- The biochemical techniques used were gelatin (for gelatinases) and transferrin zymogrphy (for MMP-3, MMP-7) and substrate assays to evaluate specific MMPs.

-----TIMPs were identified using reverse gelatin zymography and quantitated by TIMP ELISA kits or by chemiluminescent western blotting.

----- Zymographical analysis of extracts (0.1-0.2 g) of breast tissue revealed the presence of latent (92 kDa) and active (82 kDa and 62 kDa) MMP-9, latent (72 kDa) and active (62 kDa) MMP-2 and higher molecular weight aggregates of MMP-2 and -9.

-----Quantification of MMP bands showed a **general increase in MMPs** in all cancer tissues, However, the **increase in MMP-9** was the **most striking** as it was present at levels 20 times (in situ ductal) to **200 time** (in high grade **infiltrating ductal)** carcinomas compared to the low levels (often undetectable) found in **normal** tissues and **benign** tumors. MMP-9 and MMP-2 were both present in their active forms in carcinoma tissues.

-----Reverse zymography demonstrated the presence of TIMPs - 1, - 2 and - 3 in all breast tissues. Quantitation of TIMPs by ELISA and quantitation by western blotting showed a reliable trend of lower TIMPs (specifically TIMP-1) in nanogram amounts in invasive cancer tissues compared to normal and benign tissues.

-----Immunohistochemical staining of adjacent paraffin-embedded tissue sections (4 μ) with **anti-MMP-9** IgGs showed that MMP-9 was **produced or secreted by tumor cells** in high **grade infiltrating ductal carcinoma.** The red antigen complex is clearly visible in the **cytoplasmic component** of the tumor cells. Virtually no MMP-9 was seen in tissue sections of benign tumors.

-----No staining was observed for stromelysin (MMP-3) or for PUMP-1 (MMP-7) in any of the breast tissues.

-----MMP-2 staining was strongest in stroma around the tumor cells in high grade carcinoma tissue section. No staining of tumor cells was observed with anti-MMP-2 IgGs - only the stroma.

-----Anti-TIMP-1 IgGs showed weak staining in high grade carcinoma tissues -- showed 2^+ staining in normal benign tissues. Anti-TIMP-2 IgGs showed staining of stroma at almost equal intensity in all breast tissues.

-----Both zymographical and immunohistochemical studies categorically showed elevated expression of MMP-9 and under expression of TIMP-1 in high grade breast carcinoma tissue. The observed imbalance of MMPs and TIMPs in carcinoma tissues would lead to sn increased matrix degradation and contribute to the invasiveness or metastatic potential of human breast carcinomas.

-----Reverse transcription polymerase chain reaction (RT-PCR) analysis was used for semiquantitation of low-abundance mRNAs of MMPs and TIMPs gene expression in breast tissues. Although semiquantitative, the trend in the expression of genes in breast tissues may strenghten or weaken the proposed hypothesis.

-----The RT-PCR analysis showed a higher gene expression o MMP-9 in cancer tissues, the highest expression being noted in high grade infiltrating ductal carcinomas. Some benign tissued showed a low level expression of MMP-9.

-----The multipronged analysis of breast tissues categorically show the implication of MMPs, specifically MMP-9 and low expression of TIMPs specifically TIMP-1 with tumor invasion and metastasis.

(8) REPORTABLE OUTCOMES:

-----So far one manuscript and three abstracts with presentations have been generated from the four year study.

----- It was a large undertaking processing tissue extracts and looking for and quantitating not just a couple of enzymes but also the inhibitors. Immunohistochemical staining of tissues was most time consuming trying to process over 2000 tissue sections reviewed under microscope, generating photographic slides to meaningfully collate the findings.

----- It is possible to generate two-three manuscripts from this study on breast cancer.

(9) CONCLUSIONS:

The multipronged (biochemical, immunohistochemical and molecular biological) study categorically showed there is an elevated expression of MMP-9 together with overexpression of constitutively expressed MMP-2 and MMP-1; and underexpression of TIMPs -1, -2, and -3 by both stroma and tumor cells in all types of cancer. The imbalnce of destructive MMPs over their tissue inhibitors (TIMPs) may contribute to the invasiveness or metastatic potential of human breast cancer. This study also indicates that a tailor-made low molecular weight inhibitor that specifically inhibits or knocks out active MMP-9 may be invaluable therapeutic reagent to prevent the spread of breast cancer to other organs such as lung or bones.

This comprehensive study was done using the same breast tissues for the biochemical, immunohistochemical and gene expression of mRNA level analyses. This study has an advantage over many studies reported in the literature in that the evaluations of several MMPs and TIMPs were acheived in relation to each other. Mostly, the investigators choose the expression of one enzyme and its inhibitor in each disease.

The strict adherence to the statement of work demanded by the funding agency should be relaxed somewhat to pursue an interesting research finding that arise from the project.This was the case when this project was carried out. The CD44 colocalization with active MMP-9 was not stated in the statement of work and when this finding was included in the yearly report, the administration of granting agency was not pleased. The active MMP-9 colocalisation with CD44 is **very** relevant to the present study. The invadopodia movement of the cell with active MMP-9 can facilitate the movement of the tumor cell from one location to another. Again the published manuscript was the first report of the colocaliztion of MMP-9 and CD44. Subsequently, other investigators have published similar findings. CD44 is implicated in several types of cancers and its homing receptor when teamed up with an MMP would certainly facilitate the tumor cell migration. A therapeutic targeting substance against this colocalization of CD44 and MMP-9 may be of great importance in not only arresting breast cancer or other types of cancer.

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(13) FINAL REPORTS:

NAME	Personnel	Effort %
Zeenat Gunja-Smith, Ph.D.	Principal Investigator	80
Yunqui Liu	Research Specialist	100
Dr. M. Nadji	co-investigator	15

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Bourguignon, L.Y., Gunja-Smith, Z., Iida, N., Zhu, H.B., Young, L.J., Muller, W.J., and Cardiff, R.D. CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *Journal.of.Cellular Physiology.* 176(1):206-215, 1998.

Rivero, JA, **Gunja-Smith, Z**, Alamino M, Herbert L, Charyulu, VL, Lopez, DM. Signalling events involved in MMP-9 expression in T cells of mammary tumor bearing mice. AACR meeting, Philadelphia, volume 40:pg 471.1999.

JOURNAL OF CELLULAR PHYSIOLOGY 176:206–215 (1998)

APPENDIX - Z. Gunja-Smith
ITEM #1

CD44v$_{3,8-10}$ Is Involved in Cytoskeleton-Mediated Tumor Cell Migration and Matrix Metalloproteinase (MMP-9) Association in Metastatic Breast Cancer Cells

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In the present study, we have employed a unique breast cancer cell line (Met-1, which was derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene) to study the role of CD44 variant isoform(s) in the regulation of metastatic breast tumor cell behavior. The results of reverse transcriptase–polymerase chain reaction, Southern blot, nucleotide sequencing, immunoprecipitation, and immunoblot analyses indicated that these cells express a major CD44 isoform (molecular weight ≈ 260 kDa) containing a v3,8–10 exon insertion (designated as CD44v$_{3,8-10}$). In addition, we have determined that CD44v$_{3,8-10}$ binds specifically to the cytoskeletal proteins such as ankyrin. Biochemical analyses, using competition binding assays and a synthetic peptide identical to NGGNGTVEDRKPSEL (a sequence located between aa480 and aa494 of CD44v$_{3,8-10}$) indicate that this 15-amino acid peptide binds specifically to the cytoskeletal protein ankyrin (but not to fodrin or spectrin). This peptide competes effectively for ankyrin binding to CD44v$_{3,8-10}$. Therefore, we believe that the sequence ^{480}NGGNGTVEDRKPSE^{494}L, located at the cytoplasmic domain of CD44v$_{3,8-10}$, is required for the ankyrin binding. We have also detected that CD44v$_{3,8-10}$-containing Met-1 cells are capable of forming membrane spikes or "invadopodia" structures and undergo active migration processes. Treatments of Met-1 cells with certain agents including anti-CD44v$_3$ antibody, cytochalasin D (a microfilament inhibitor), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule disrupting agent) effectively inhibit "invadopodia" formation and subsequent tumor cell migration. Further analyses using zymography assays and double immunofluorescence staining indicated that CD44v$_{3,8-10}$ is closely associated with the active form of matrix metalloproteinase, MMP-9, in a complex within "invadopodia" structures. These findings suggest that CD44v$_{3,8-10}$ plays an important role in linking ankyrin to the membrane-associated actomyosin contractile system required for "invadopodia" formation (coupled with matrix degradation activities) and tumor cell migration during breast cancer progression. J. Cell. Physiol. 176:206–215, 1998. © 1998 Wiley-Liss, Inc.

CD44 isoforms belong to a family of glycoproteins that are expressed in a variety of cells and tissues (Lesley et al., 1993; Bourguignon, 1996). The external domain of certain CD44 isoforms is known to mediate both cell adhesion to extracellular matrix (ECM) components (e.g. hyaluronic acid, fibronectin, and collagen) and homotypic cell aggregation (Stamenkovic et al., 1991; Lesley et al., 1992, 1993; Underhill, 1992; Yang et al., 1994). The cytoplasmic domain of the CD44 iso-

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forms contains a specific binding region required for ankyrin binding (Bourguignon, 1996; Lokeshwar et al., 1996). Posttranslational modification of the cytoplasmic domain of CD44 by protein kinase C (Kalomiris and Bourguignon, 1989), acylation (Bourguignon et al., 1991), or GTP binding (Lokeshwar and Bourguignon, 1992) has been found to enhance the binding between CD44 and ankyrin. These observations suggest that CD44 not only functions as an adhesion protein but also may play an important role in signaling cytoskeleton-mediated cellular activities (Bourguignon et al., 1993; Bourguignon, 1996).

It is now known that all CD44 isoforms are encoded by a single gene that contains 19 exons (Screaton et al., 1992). Of the 19 exons, 12 exons can be alternatively spliced (Arch et al., 1992; Screaton et al., 1992; Tolg et al., 1993). Most often, alternative splicing occurs between exons 5 and 15, leading to an insertion in tandem of one or more variant exons within the membrane proximal region of the extracellular domain (Screaton et al., 1992). The variable amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations (Lokeshwar and Bourguignon, 1991; Bourguignon, 1996; Lokeshwar et al., 1996) and glycosaminoglycan (GAG) additions (Bennett et al., 1995). For example, one of the CD44 isoforms (so-called $CD44v_3$) contains the v3 (or exon 7) insertion that contains heparan sulfate addition sites. It has been suggested that this molecule binds a wide range of heparin binding growth factors, cytokines, and chemokines (Bennett et al., 1995).

It is clear that the mechanisms responsible for tumor progression are very complex, involving a number of biochemical and cellular events (Hung, 1988; Zetter, 1990). In human cells, matrix metalloproteases (MMPs), such as MMP-2 (72 kDa), MMP-9 (92 kDa), and MMP-3 (57 kDa), are most likely involved in the initial degradation of the basement membrane surrounding the tumor (Liotta, 1984), which is required for tumor invasion and metastasis (Boyd, 1996). Recent data indicating colocalization of MMP-2 and integrin $\alpha v\beta 3$ on the surface of invasive melanoma cells and blood vessels (Brooks et al., 1996) suggest that there is a close interaction between MMPs and certain adhesion molecules (e.g., integrins) during tumor invasion and/or metastasis. The $CD44v_3$-containing isoforms, another class of adhesion molecule, are also preferentially expressed on the surface of metastatic tumor cells during the progression of human breast carcinomas (Bourguignon et al., 1995; Iida and Bourguignon, 1995). The question of whether there is an interaction between MMPs and $CD44v_3$-containing isoforms in metastatic breast tumor cells is addressed in the present study.

We have employed a unique breast cancer cell line (Met-1, which was derived from high metastatic potential tumors in transgenic mice expressing polyomavirus middle T oncogene) to study the role of $CD44v_3$-containing isoforms in regulating metastatic breast tumor cell behavior. Using a variety of techniques (including biochemical, immunocytochemical, and molecular biological approaches), we have found that the $CD44v_{3,8-10}$ isoform is involved in a number of metastatic tumor cell behaviors including invadopodia formation, matrix degradation, and tumor cell migration during breast cancer progression.

MATERIALS AND METHODS

Cell culture

Mammary tumor cells containing the polyoma virus middle T (PyV-MT) transgene under the transcriptional control of the MMTV LTR promoter were used to initiate a transplantable line in nude mice. The PyV-MT transgenic mammary tumor cells were obtained from mammary tumors that arose in the transgenic colony at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario, Canada; Dr. Willliam J. Muller; Guy et al., 1992). Mammary tumors were treated with collagenase/dispase (Worthington, Freehold, NJ), and 5×10^5 cells per 100 μl were transplanted subcutaneously as a bolus via syringe and a 25-gauge needle into the thoracic region of nude mice. The resulting high potential metastatic PyV-MT transgenic mammary tumor line, Met-1, was maintained by serial tansplantation of 1-mm^3 tumor segments into either subcutaneous tissue (ectopic) or intact mammary fat pads (orthotopic).

The Met-1 tumor line was dissociated after transplant generation one and plated onto T-75 flasks to develop a tissue culture line (Young et al., 1995; Cheung et al., 1997). The Met-1 cell line was cultured in high glucose DMEM supplemented by 10% fetal bovine serum, 2 mM glutamine, and antibiotics (Sigma, St Louis, MO). The Met-1 cell line is currently in passage 30.

Immunoreagents

Monoclonal rat anti-human CD44 antibody (clone 020, isotype IgG_{2b}; obtained from CMB-TECH, Miami, FL) used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s and other variant isoforms (Chaitin et al., 1994; Iida and Bourguignon, 1995) and is capable of precipitating all CD44 variants. For the preparation of polyclonal rabbit anti-CD44v3 or rabbit anti-MMP-9, specific synthetic peptides ($\approx$15–17 amino acids unique for either CD44v3 sequences or MMP-9 sequences, e.g., the metal binding region peptide of MMP-9) were prepared by the Peptide Laboratories of the Department of Biochemistry and Molecular Biology using an Advanced Chemtech automatic synthesizer (model ACT350). Conjugated CD44v3 or MMP-9 peptides (to hemocyanin or polylysine) were injected into rabbits to raise the antibodies. The anti-CD44v3 or anti-MMP-9 sera were collected from each bleed and stored at 4°C containing 0.1% azide. Both rabbit anti-CD44v3 and rabbit anti-MMP-9 IgGs were prepared by using conventional DEAE-cellulose chromatography (Campbell et al., 1974) and were tested to be monospecific (by immunoblot assays).

Metabolic labeling procedures

Confluent Met-1 cells ($\approx 1 \times 10^6$/60-mm dish) were incubated with sulfate-free Joklik medium for 6 hr, followed by $(^{35}SO_4)^{2-}$ labeling (300 μCi/ml) for 4 hr at 37°C. These radioactive sulfate-labeled cells were washed in phosphate buffered saline, pH 7.2 (PBS), and solubilized in RIPA buffer (Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1996). Subsequently, these ^{35}S-labeled cells were used for anti-CD44-mediated immunoprecipitation as described below.

Cell surface labeling procedures

Met-1 cells suspended in PBS were surface labeled by using the following biotinylation procedure. Briefly, cells (10^7 cells/ml) were incubated with sulfosuccinomidobiotin (Pierce, Rockford, IL; 0.1mg/ml) in labeling buffer (150 μM NaCl, 0.1 M HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44-mediated immunoprecipitation, as described previously (Bourguignon et al., 1997). These biotinylated materials precipitated by anti-CD44 antibody were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to the nitrocellulose filters, and incubated with ExtrAvidin-peroxidase (Sigma). After an addition of peroxidase substrate (Pierce), the blots were developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturer's instructions.

Immunoprecipitation and immunobloting techniques

Unlabeled or labeled (surface biotinylated or radioactive sulfate labeled) cells were washed in 0.1 M PBS and solubilized in RIPA buffer (Lokeshwar and Bourguignon, 1991; Lokeshwar et al., 1996). The solubilized extracts were incubated with rat anti-CD44 antibody at 4°C for 15 hr, followed by incubation with goat anti-rat IgG agarose beads at 4°C for 90 min, as described previously (Lokeshwar and Bourguignon, 1991; Lokeshwar et al., 1996). The immunoprecipitates were analyzed by 7.5% SDS-PAGE followed by fluorography. In some cases, Met-1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rat anti-CD44 antibody followed by goat anti-rat IgG. The immunoprecipitated material was solubilized in SDS, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-CD44v3 antibody (5 μg/ml) or rabbit anti-MMP-9 antibody (5 μg/ml) for 1 hr at room temperature, followed by incubation with horse radish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 hr. The blots were developed using Renaissance chemiluminescence reagent (NEN, DuPont, Boston, MA) according to the manufacturer's instructions.

Double immunofluorescence staining

To detect "invadopodia" formation (Mueller and Chen, 1991; Monsky et al., 1994), Met-1 cells were first washed with PBS (0.1 M phosphate buffer, pH 7.5, and 150 mM NaCl) buffer and fixed by 2% paraformaldehyde. In some cases, cells were pretreated with various agents (e.g., rabbit anti-$CD44v_3$ antibody, preimmune rabbit IgG, 20 μg/ml cytochalasin D, 20 μM W-7, or 1×10^{-5} M colchicine) at room temperature for 30 min, followed by fixing with 2% paraformaldehyde. Subsequently, cells were incubated with fluorescein-conjugated rat anti-CD44 antibody (50 μg/ml) or fluorescein-conjugated rat IgG (50 μg/ml; as a negative control). These fluorescein-labeled cells were then rendered permeable by ethanol treatment and stained with rhodamine-conjugated rabbit anti-MMP-9 IgG. To detect nonspecific antibody binding, fluorescein-labeled cells were incubated with rhodamine-conjugated preimmune rabbit IgG. No labeling was observed in such control samples.

The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Binding of ^{125}I-labeled ankyrin/fodrin/spectrin to synthetic peptide and scrambled peptides

Nitrocellulose disks (1 cm in diameter) were coated with ≈1 μg of either the ankyrin-binding region peptide (NGGNGTVEDRKPSEL) or a scrambled peptide (GRNETNPEGSGLDVK; synthesized by Dr. Eric Smith, University of Miami) at 4°C for 16 hr. After coating, the unoccupied sites on the disks were blocked by incubation with a solution containing 20 mM Tris-HCl, pH 7.4, and 0.3% bovine serum albumin at 4°C for 2 hr. The disks were then incubated with ^{125}I-labeled ankyrin/spectrin/fodrin (≈3,000 cpm/ng) at 4°C for 2 hr in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% bovine serum albumin). After binding, the disks were washed three times in the binding buffer and the disk-bound radioactivity was estimated. The nonspecific binding was determined in the presence of a 100-fold excess of one of the respective unlabeled ligands and subtracted from the total binding. Nonspecific binding was approximately 30% of the total binding. As a further control, the ligands were also incubated with uncoated nitrocellulose disks to determine the binding observed due to the "stickiness" of various ligands. Nonspecific binding was observed in these controls.

Binding of ^{125}I-labeled $CD44v_{3,8-10}$ to ankyrin

Purified ^{125}I-labeled $CD44v_{3,8-10}$ (≈0.32 nM protein, 1.5×10^4 cpm/ng) was prepared according to the procedures described previously (Bourguignon et al., 1993) and incubated with 30 μl of ankyrin conjugated to sepharose beads (≈0.75 μg protein) in 0.5 ml of the binding buffer (described above). Binding was carried out in the presence or absence of various concentrations (1 nM to 1 μM) of unlabeled competing synthetic peptide (NGGNGTVEDRKPSEL, corresponding to the ankyrin binding sequence of CD44) (Lokeshwar et al., 1994) at 4°C for 5 hr under equilibrium conditions. Equilibrium conditions were determined by performing a time course (e.g., 1–10 hr) of the binding reaction. After binding, the beads were washed in the binding buffer and the bead-bound radioactivity was determined. Nonspecific binding was determined in the presence of either a 100-fold excess of unlabeled ankyrin or with bovine serum albumin-conjugated sepharose beads. Nonspecific binding was approximately 20–30% of the total binding and was subtracted from the total binding.

Reverse transcriptase–polymerase chain reaction (RT-PCR) and Southern blot analysis

Total RNA was extracted from Met-1 cells by using the method described previously (Chomczynski and Sacci, 1987). Approximately 3 μg of total RNA were used to synthesize first-strand oligo (dT)-primed cDNA at 42°C for 1 hr by an RT system (Promega, Madison,

WI) containing avian myeloblastosis virus RT. After first-strand synthesis, PCR amplification of cDNA was carried out with an initial melting of the RNA/cDNA hybrid at 94°C for 30 sec, annealing at 60°C for 30 sec, and polymerization at 72°C for 1 min. One pair of PCR primers was used to amplify between exon 5 and v3 (exon 7) to detect any splice variants of CD44 mRNAs containing v1, v2, or v3. Another pair was designed to amplify between v3 (exon 7) and exon 15 to detect CD44 mRNAs containing v3–10. The PCR primers used in this study were as follows: CD44 exon 5 left primer (5′-ACATCAGTCACAGACCTGCCC-3′), CD44 exon 15 right primer (5′-ATCCATGAGTCACAGTGCGG-3′), CD44v3 (exon 7) right primer (5′-CTGAGGTGTCTG-TCTCTTTC-3′), and CD44v3 (exon 7) left primer (5′-GACTCCACAGACAGAGAAAG-3′). A negative control was carried out in the absence of RT, and a positive control was carried out using human CD44v (v3, v8, v9, v10) cDNA as a template. The PCR products were examined on 2.0% agarose gel followed by Southern blot analysis hybridizing with human CD44 cDNA and/or mouse CD44 cDNA probes using the protocol previously described (Iida and Bourguignon, 1995). The PCR products were also one-step cloned using a TA-cloning kit (Invitrogen, San Diego, CA) and sequenced by the dideoxy sequencing method (Iida and Bourguignon, 1995).

Zymography and immunoprecipitation

Gelatin zymography was used with a modified procedure by Herron et al., 1986; Gunja-Smith et al., 1996) for detecting picograms of MMP-2 and MMP-9 and nanograms of other MMPs and proteases. SDS-PAGE was performed in 7.5% or 10% polyacrylamide containing 0.33 mg/ml gelatin. The gels were then rinsed twice in 0.25% Triton X-100 and incubated in the assay buffer [0.05 M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN_3, and 0.005% Brij 35] at 37°C for 18 hr. Gels were then stained with Coomassie blue R250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. Each gel lane contained preestimated amount of MMPs.

Immunoprecipitation analysis was performed according to standard methods. Serum-free media, Met-1 membrane fractions (Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1996), or anti-CD44-associated immune complexes were collected and analyzed for gelatinases or other MMP(s) content by zymography or substrate assays. In addition, conditioned serum-free media or Met-1 membrane fractions containing MMP(s) were immunoprecipitated with monospecific rabbit anti-MMP-9 IgG(s) using protein A-agarose suspensions (50–95 μg of protein A) at 4°C. A negative control, normal rabbit IgG or preimmune serum (in which endogenous MMPs, especially gelatinases, were removed by gelatin-sepharose affinity columns) was used. After the reacted agarose gels were washed three times with buffer (0.05M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, 3 mM PMSF, 0.02% NaN_3, and 0.005% Brij 35), the immune complexes were analyzed by zymography for specific enzyme activity or for proteins by SDS-PAGE.

The Met-1 membrane fraction or conditioned serum-free media were also used to quantitate for the MMP-2 or MMP-9 gelatinase (MMPs) activity using ^{3}H-acetylated type I gelatin (Sellers et al., 1978; Gunja-Smith et al., 1996). Assays were set up in the presence or absence of 1.0 mmol/L aminopheylmercuric acetate (APMA) to measure latent and active enzymes; negative control samples containing 2 mmol/L 1,10-phenanthroline were also run in the assays.

Tumor cell migration assays

Twenty-four transwell units were used for monitoring in vitro cell migration, as described previously (Merzak et al., 1994). Specifically, the 8-μm porosity polycarbonate filters were used for the cell migration assay (Merzak et al., 1994). Met-1 cells [$\approx 1 \times 10^4$ cells/well in PBS, untreated or treated with various agents such as 20 μg/ml cytochalasin D, 20 μM W-7, 1×10^{-5} M colchicine, or 50 μg/ml rabbit anti-CD44v_3 antibody) were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented with 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18 hr of incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters by standard cell number counting methods, as described previously (Merzak et al., 1994). Each assay was performed in triplicate and repeated at least three times. All data were analyzed statistically by Student's t-test, and statistical significance was set at $P < 0.01$.

RESULTS

Expression of CD44 variant isoform(s) in Met-1 cells

The expression of CD44 variant isoforms is closely correlated with metastatic and proliferative behaviors of a variety of tumor cells including various carcinomas such as human breast tumor cells (Bourguignon et al., 1995; Iida and Bourguignon, 1995). To examine directly the CD44 isoform expression on the surface of Met-1 cells, we have used surface biotinylation techniques and a specific monoclonal anti-CD44 antibody that recognizes the CD44 epitope located at the N-terminus of the common domain (Iida and Bourguignon, 1995; Lokeshwar et al., 1996). Our results indicate that a single surface-biotinylated polypeptide (molecular weight $\approx$ 260 kDa) displaying immunological cross-reactivity with CD44 is preferentially expressed on the surface of Met-1 (Fig. 1A). This surface-labeled 260-kDa protein can also be immunoprecipitated (Fig. 1B) or immunoblotted (Fig. 1D) by rabbit anti-CD44v_3 antibody raised specifically against the v_3 sequence, suggesting that this protein is a CD44v_3-containing isoform. No CD44v_3-containing material is observed in control samples when either normal rat IgG or preimmune rabbit serum is used (Fig. 1F).

To verify the expression of CD44v_3-containing transcript(s) at the RNA level, total RNA from Met-1 cells was extracted and analyzed by RT-PCR using exon specific primers. Using a PCR primer pair to amplify between v3 (exon 7) and exon 15 by RT-PCR (Fig. 2C-a), a single product is detected at approximately 530 bp (Fig. 2A, lane 1). The size of the amplified fragment appears to correspond to a known CD44 variant isoform

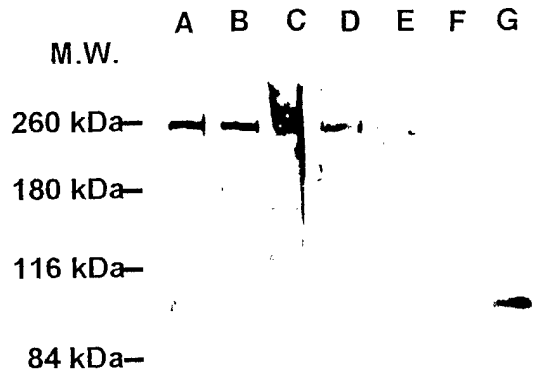

Fig. 1. Characterization of CD44 expression in Met-1 cells. **A:** Immunoprecipitation of surface biotinylated Met-1 cells using monoclonal anti-CD44 antibody (which recognizes the CD44 epitope located at the N-terminus of the common domain). **B:** Immunoprecipitation of surface biotinylated Met-1 cells with rabbit anti-CD44v_3-specific antibody. **C:** Immunoprecipitation of Met-1 cells (metabolically labeled with $(^{35}SO_4)^{2-}$ with rabbit anti- CD44v_3-specific antibody. **D:** Immunoblot of Met-1 cells with rabbit anti-CD44v_3-specific antibody. **E:** Coimmunoprecipitation of MMP-9 with CD44: immunoprecipitation of Met-1 cells with rabbit anti-MMP-9 followed by immunoblot with rat anti-CD44 (which recognizes the CD44 epitope located at the N-terminus of the common domain). **F:** Immunoprecipitation of surface biotinylated Met-1 cells with normal preimmune rabbit serum (or normal rat IgG; data not shown). **G:** Immunoblot of Met-1 cells with rabbit anti-MMP-9 antibody.

that contains v3 and v8–10 exon insertions. Using a PCR primer pair to amplify between exon 5 and v3 (exon 7; Fig. 2C-b), one amplified DNA fragment is observed (about 270 bp, as shown in Fig. 2B, lane 1) that is identical to an amplimer from a human variant CD44 cDNA template containing v3, v8–10 exons (Fig. 2B, lane 2). The RT-PCR reaction is specific because no amplified fragment can be detected in samples without any RT (Fig. 2A, lane 2, 2B, lane 3). This v3-containing PCR product was subsequently "one-step cloned" into the pCR™ vector from Invitrogen and sequenced. Our nucleotide sequence data indicate that only v3 (but not v1 or v2) is present in CD44 transcripts expressed in Met-1 cells and that the variant exon structure of CD44 transcripts containing v3 in Met-1 cells is the CD44$v_{3,8-10}$ isoform (Fig. 2C-c). This CD44$v_{3,8-10}$ variant exon structure was previously identified in human breast carcinoma samples (Bourguignon et al., 1995; Iida and Bourguignon, 1995) and its molecular mass (expressed at the protein level) has been shown to be ≈260 kDa (Bennett et al., 1995). Therefore, we believe that the major CD44v_3-containing isoform expressed on the surface of Met-1 cells is the CD44$v_{3,8-10}$ isoform.

A number of CD44 isoforms have been shown to contain sulfated oligosaccharides (Bennett et al., 1995; Lokeshwar et al., 1996). Consequently, we metabolically labeled Met-1 cells with $^{35}SO_4^{2-}$ and then looked for the presence of radioactive sulfate label in CD44$v_{3,8-10}$ using anti-CD44v3-mediated immunoprecipitation (Fig. 1C). Our results indicate that $^{35}SO_4^{2-}$ is incorporated into CD44$v_{3,8-10}$ (possibly at the GAG chains; Fig. 1C). The GAG chains of certain CD44v_3-containing isoforms may be involved in the binding of heparin binding growth factors, as shown previously (Bennett et al., 1995).

Transmembrane interaction between CD44$v_{3,8-10}$ and ankyrin

Previously, it has been shown that the ankyrin binding sequence (e.g., 15aa-NGGNGTVEDRKPSEL) in the cytoplasmic domain of CD44 is conserved in most of the CD44 isoforms and is clearly involved in the ankyrin binding (Lokeshwar et al., 1994). To establish that this 15aa region of CD44$v_{3,8-10}$ is directly involved in the ankyrin binding, we tested the ability of a synthetic peptide (NGGNGTVEDRKPSEL, corresponding to the 15aa sequence between aa480 and aa494 of CD44$v_{3,8-10}$) to bind various cytoskeletal proteins. This synthetic peptide binds to ankyrin specifically and does not bind a scrambled peptide (GRNETNPEGSGLDVK) or other cytoskeletal proteins such as fodrin and spectrin (Table 1). Furthermore, we used competition binding assays and a synthetic peptide identical to the ankyrin binding domain (e.g., NGGNGTVEDRKPSEL) to analyze the ankyring binding of CD44$v_{3,8-10}$. Our results indicate that the synthetic peptide corresponding to the ankyrin binding domain of the CD44 isoform competes effectively with CD44$v_{3,8-10}$ in binding to ankyrin with an apparent inhibition constant (K_i) of approximately 0.7 nM (Fig. 3). These findings strongly suggest that an interaction between CD44$v_{3,8-10}$ and the cytoskeleton occurs at the cytoplasmic region of CD44$v_{3,8-10}$ (i.e., ^{480}NGGNGTVEDRKPSE^{494}L) in Met-1 tumor cells.

Furthermore, we found that Met-1 cells are capable of forming membrane spikes or "invadopodia" structures (Fig. 5). Using in vitro migration assays, we found that CD44$v_{3,8-10}$- containing Met-1 cells undergo active cell migration (Table 2). Treatments of Met-1 cells with various agents such as anti-CD44v_3 antibody, cytochalasin D (a microfilament-disrupting agent that prevents actin polymerization), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule inhibitor) (Table 2) cause a significant inhibition of "invadopodia" formation and subsequent tumor cell migration (Table 2). These findings suggest that CD44$v_{3,8-10}$ and associated microfilament components play an important role in the regulation of both "invadopodia" formation and tumor cell motility.

Association of CD44$v_{3,8-10}$, MMPs, and MMP-9 in Met-1 cells

Metastatic tumor cells are capable of degrading the ECM barrier to migrate out of the primary tumor location and into the circulation to establish a site of metastasis (Merzak et al., 1994; Boyd, 1996). The breakdown of the ECM can be traced to the action of one or more MMPs that belong to a family of zinc proteases (Woessner, 1991; Matrisian, 1992; Nagase, 1996). Normally, they are secreted as proenzymes and become activated outside the cell by serine proteases, such as plasmin, by the removal of a 9-kDa prosegment from the active site. Several MMPs, such as the 72-kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92-kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the 57-kDa stromelysin (MMP-3), and interstitial collagenase (MMP-1), are likely to be responsible for ECM degradation during tumor invasion and metastasis (Stetler-Stevenson et al., 1993; Boyd, 1996).

In the present study, we employed a number of techniques, including zymographic assays, immunoblot/im-

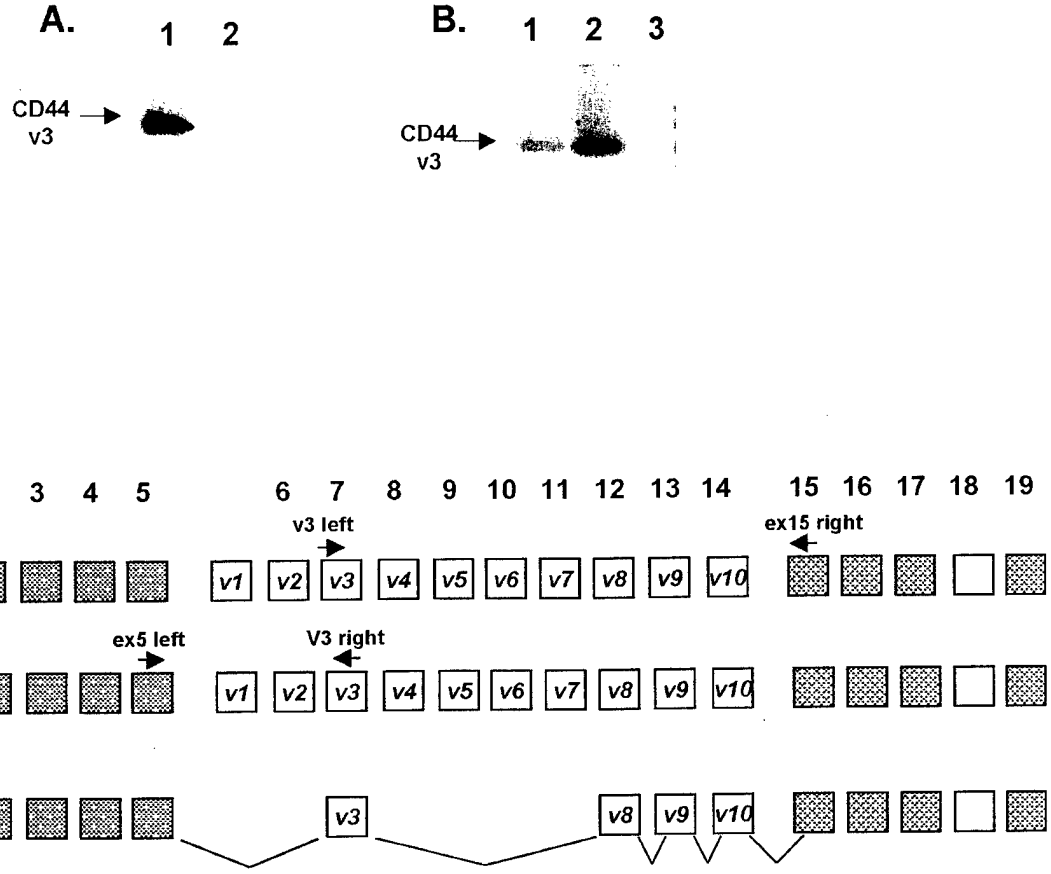

Fig. 2. RT-PCR and Southern blot analysis of CD44 transcript expression in Met-1 cells. Total RNA isolated from Met-1 cells were reverse-transcribed and subjected to PCR by using PCR primer pairs, as described in C-a and C-b. Subsequently, RT-PCR products were analyzed by Southern blot hybridization, cloning, and nucleotide sequence analysis as described in Materials and Methods. **A:** Southern blot analysis of a single RT-PCR product (≈530 bp) generated by $CD44v_3$ (exon 7) and exon 15 primer pairs (the primer design is shown in C-a, lane 1). As a negative control, RT-PCR was carried out in the absence of RT (lane 2). **B:** Southern blot analysis of a single RT-PCR product (≈270 bp) generated by CD44 exon 5 and v3 (exon 7) primer pairs (the primer design is shown in C-b, lane 1). As a positive control, a known CD44 variant isoform that contains v3 and v8–10 exon insertions was used (lane 2). As a negative control, RT-PCR was carried out in the absence of RT (lane 3). **C:** Schematic exon map of CD44 in mouse. **C-a:** Specific $CD44v_3$ (exon 7) and exon 15 primer pairs used in A. **C-b:** Specific CD44 exon 5 and v3 (exon 7) primer pairs used in B. **C-c:** Schematic illustration of the $CD44v_{3,8-10}$ isoform identified in Met-1 cells.

TABLE 1. Binding (nM × cpm bound) of ^{125}I-labeled cytoskeletal proteins (ankyrin, spectrin, and fodrin) to synthetic peptides[1]

Binding to NGGNGTVEDRKPSEL	
^{125}I-labeled ankyrin	55,500 ± 105
^{125}I-labeled spectrin	1,020 ± 32
^{125}I-labeled fodrin	1,100 ± 42
Binding to GRNETNPEGSGLDVK	
^{125}I-labeled ankyrin	1,150 ± 28
^{125}I-labeled spectrin	1,090 ± 21
^{125}I-labeled fodrin	1,178 ± 30

[1]The ^{125}I-labeled cytoskeletal proteins (100 ng each of ankyrin, spectrin, and fodrin) were incubated with nitrocellulose disks coated with either the synthetic peptide NGGNGTVEDRKPSEL (corresponding to the sequence between aa480 and 494 of human CD44s) or the scrambled peptide GRNETNPEGSGLDVK at 4°C for 4 hr as described in Materials and Methods. Nonspecific binding was determined in the presence of a 100-fold excess of the respective unlabeled cytoskeletal proteins and subtracted from the total binding.

munoprecipitation techniques, and immunofluorescence staining to examine the relationship between gelatinase activities and the $CD44v_{3,8-10}$ in Met-1 tumor cells. The results of the gelatin zymographic analyses indicate that the lytic band of mouse gelatinase in the membrane fraction of Met-1 cells is approximately 92 kDa (Fig. 4A-a). No other lytic bands of gelatinase were observed. Using anti-human MMP-9-mediated immunoblot (Fig. 1G) and immunoprecipitation (Fig. 4A-b) of Triton X-100 solubilized Met-1 membranes, we found that this 92-kDa protein displays lytic gelatinase activities corresponding to the lytic band of human MMP-9 (Fig. 4B-d). As a control, the zymographic profile of anti-MMP-9 IgG alone (without using Triton X-100 solubilized Met-1 membranes and immunoprecipitation) shows no lytic bands of gelatinase (Fig. 4A-d). This result suggests that the lytic band of 92-kDa gelatinase detected in the membrane fraction of Met-1 cells (Fig. 4A-b) corresponds to a MMP-9-like molecule.

It has been determined that the lytic band corresponding to the latent form of normal mouse MMP-9 is approximately 105 kDa (Reponen et al., 1994; Tanaka et al., 1993; Fig. 4B-a). Our data indicate that the 92-kDa gelatinase from Met-1 membrane fraction

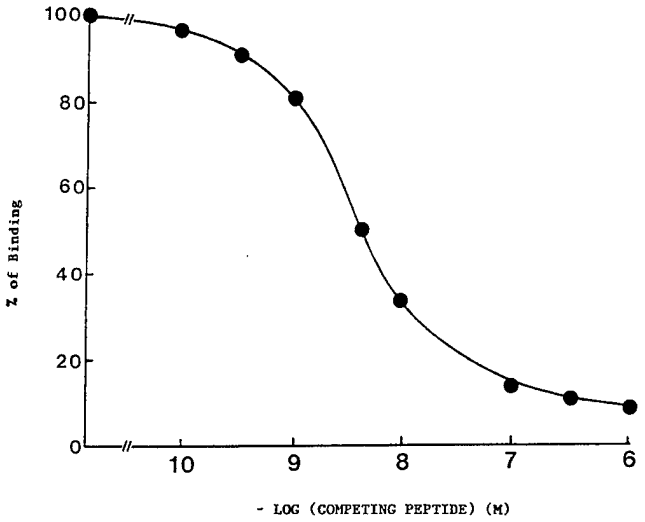

Fig. 3. Binding of ^{125}I-labeled CD44v$_{3,8-10}$ to ankyrin. ^{125}I-labeled CD44v$_{3,8-10}$ was incubated with ankyrin in the presence of different concentrations of unlabeled synthetic peptide (NGGNGTVEDRKP-SEL) corresponding to the ankyrin binding domain of CD44 as described in Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

TABLE 2. Effects of different drugs on "invadopodia" formation and cell migration

Treatments[1]	"Invadopodia" (% of control)[2]	Cell migration[3]
No treatment (control)	100	100
Preimmune serum	96	98
Anti-CD44v$_3$ antibody	15	23
Cytochalasin D	28	22
W-7	18	16
Colchicine	98	97

[1]The concentrations of reagents used in this experiment were 50 μg/ml rabbit anti-CD44v$_3$, 20 μg/ml cytochalasin D, 20 μM W-7, and 1×10^{-5} M colchicine.
[2]The values expressed represent an average of triplicate determinations of 3–5 experiments with a standard deviation of less than ±5%.
[3]In these experiments, ≈30–40% of input cells (≈1×10^4 cells/well) underwent in vitro migration in the control samples.

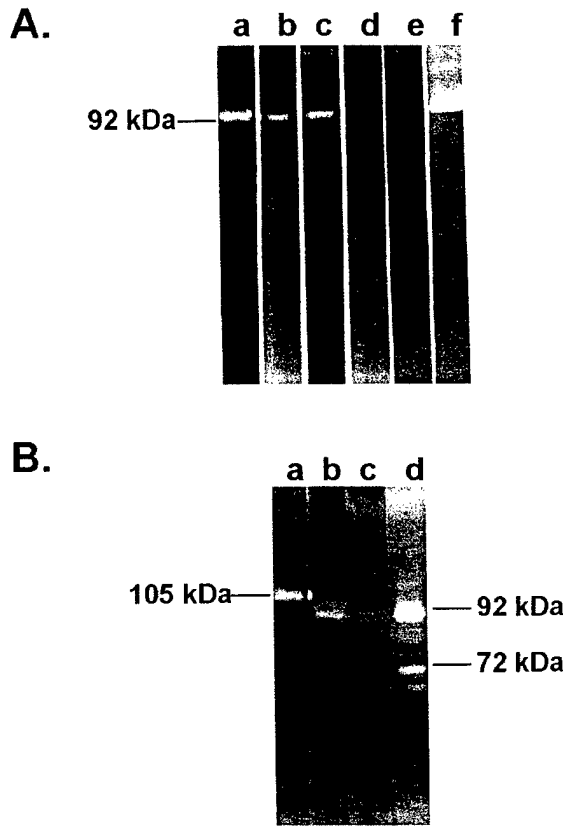

Fig. 4. Gelatin zymographic analysis of MMPs in Met-1 cells. Gelatin zymography was used to detect MMPs in Met-1 cells according to the procedures described in Materials and Methods. **A-a:** The 92-kDa lytic gelatinase band detected in Met-1 membranes. **A-b:** The human MMP-9 (92-kDa lytic gelatinase band) detected by anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes. **A-c:** The 92-kDa lytic gelatinase band associated with the anti-CD44-immunoprecipitated materials. **A-d:** As a control, anti-MMP-9 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation). **A-e:** As a control, anti-CD44 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation). **A-f:** The secreted form of MMP-9 (containing both latent, 105 kDa, and active, 92 kDa, mouse gelatinase) collected from the serum free conditioned media containing Met-1 cells. **B-a:** The lytic band (≈105 kDa) corresponding to the latent form of normal mouse MMP-9. **B-b:** The 92-kDa gelatinase from Met-1 membrane fraction. **B-c:** Treatment of the Met-1"membrane fraction with alpha(2)-macroglobulin (an inhibitor known to inactivate metalloprotease activity). **B-d:** Human MMP-9 (92 kDa) and MMP-2 (72 kDa) markers.

appears to be slightly smaller than the normal latent form of mouse MMP-9 (105 kDa; Fig. 4B-b). The reduced molecular weight of MMP-9 of Met-1 suggests that this gelatinase is in an active form. Treatment of the Met-1 membrane fraction with alpha(2)-macroglobulin (an inhibitor that binds active metalloprotease activity) (Nagase et al., 1994) results in blockage of the lytic reaction of 92-kDa gelatinase activity (Fig. 4B-c). Measurement of the gelatinase activity using ^{3}H-acetylated type I gelatin (Sellers et al., 1978; Gunja-Smith et al., 1996) indicates that the MMP-9 associated with Met-1 membranes is an active gelatinase. This enzyme does not require APMA to become active, and its activity can be readily inhibited by 1,10-phenanthroline (data not shown). These findings further support the notion that the lytic band of the 92-kDa mouse MMP-9 associated with Met-1 membranes is present in a proteolytically active form. In addition, we observed a broad lytic band (possibly a secreted form of MMP-9) in the serum-free conditioned media from Met-1 cells, which may represent the lytic bands of both the latent (105 kDa) and active (92 kDa) mouse gelatinase (Fig. 4A-f).

Further analyses by double immunofluorescence labeling and confocal microscopy indicate that the 92-kDa mouse MMP-9-like molecule (Fig. 5B) is colocalized with CD44v$_{3,8-10}$ (Fig. 5A) in the "invadopodia" structure of the Met-1 cells. No CD44v$_{3,8-10}$ or MMP-9 staining was observed in control samples when either normal rat IgG (Fig. 5a) or preimmune rabbit IgG (Fig. 5b) was used. To explore the possible association between these two molecules, we carried out anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes followed by immunoblot-

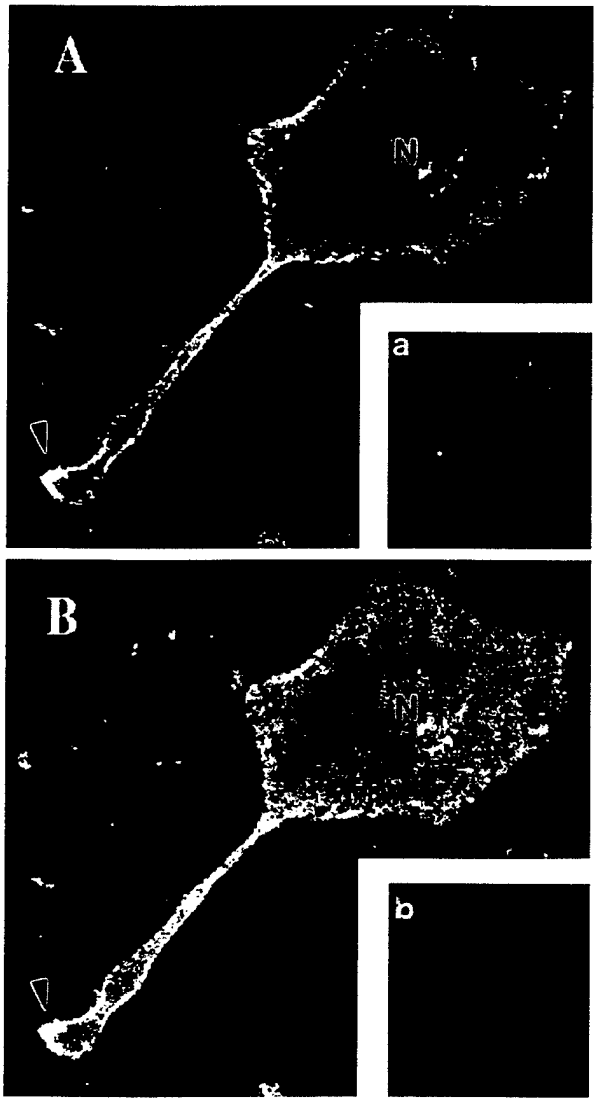

Fig. 5. Double immunofluorescence staining and confocal analysis of "invadopodia" structures (arrowheads) in Met-1 cells. **A:** Immunostaining of surface CD44v$_{3,8-10}$ using fluorescence-labeled rat anti-CD44 IgG. **a:** A negative control of surface CD44v$_{3,8-10}$ staining using fluorescence-labeled normal rat IgG. **B:** Immunostaining of intracellular MMP-9 using rhodamine-labeled rabbit anti-MMP-9 IgG. **b:** A negative control of intracellular MMP-9 staining using rhodamine-labeled preimmune rabbit IgG.

ing with anti-CD44v$_3$ antibody. Our data show that CD44v$_{3,8-10}$ is coprecipitated with the metalloprotease (e.g., MMP-9) as a complex (Fig. 1E). In addition, we carried out anti-CD44-mediated precipitation followed by gelatin zymography analysis. Our results indicate that the 92-kDa lytic gelatinase band can be detected in the anti-CD44-immunoprecipitated materials (Fig. 4A-c). As a control, the zymographic profile of anti-CD44 IgG (without using Triton X-100 solubilized Met-1 membranes and immunoprecipitation) shows no lytic bands of gelatinase (Fig. 4A-e). These findings suggest that CD44v$_{3,8-10}$ and 92 kDa are tightly associated with each other.

DISCUSSION

Several studies have indicated that the expression of CD44 on the cell surface changes profoundly during tumor metastasis, particularly during the progression of various carcinomas (Arch et al., 1992; Bourguignon et al., 1995; Iida and Bourguignon, 1995). Many tumor cells and tissues have been found to express different CD44v isoforms (Arch et al., 1992; Bourguignon et al., 1995; Droll et al., 1995; Iida and Bourguignon, 1995). These isoforms, containing additional exon insertions (e.g., exons 6–11) within the CD44 membrane proximal region, appear to be associated with tumor formation (Iida and Bourguignon, 1997) and metastasis (Arch et al., 1992; Bourguignon et al., 1995; Iida and Bourguignon, 1995). In fact, certain CD44v expression has been used as an indicator of metastasis. In the present study, we chose Met-1 cells, which were derived from high metastatic potential tumors, as a model system to analyze the relationship between CD44 isoform expression and breast tumor progression. Immunological analyses indicated that the CD44 isoform (≈260 kDa) expressed in Met-1 displays strong cross- reactivity with several anti-CD44 antibodies including rat anti-human CD44 antibody (clone 020; Fig. 1A), rabbit anti-CD44v3 antibody (Fig. 1B), and two other anti-mouse CD44 antibodies (e.g., IM-7 and IRAWB-14; data not shown). Further analyses using RT-PCR, Southern blot, and nucleotide sequence techniques indicated that this 260-kDa CD44 isoform contains a v$_{3,8-10}$ exon insertion. CD44v$_3$-containing isoforms, including CD44v$_{3,8-10}$, have been shown to be preferentially expressed on highly malignant breast carcinoma tissues but not on normal or noninvasive breast cancer tissues. In fact, there is a direct correlation between CD44v$_{3,8-10}$ isoform expression and increased histologic grade of the malignancy (Iida and Bourguignon, 1995; Sinn et al., 1995; Kalish et al., 1997). These findings suggest that breast tumor expression of the CD44v$_3$ isoform may be used as an accurate predictor of overall survival (e.g., nodal status, tumor size, and grade; Iida and Bourguignon, 1995; Kalish et al., 1997; Kaufmann et al., 1995). Therefore, it is not surprising that CD44v$_{3,8-10}$ was the predominant CD44 species detected on the surface of these highly metastatic Met-1 cells. Because information regarding the role of CD44v$_{3,8-10}$ in regulating mammary tumor cell function is very limited at the present time, it is clearly important to examine the structural properties of this molecule.

CD44v$_{3,8-10}$ (with heparan sulfate, or GAG, addition) has been shown to bind bFGF on COS cells (Bennett et al., 1995). Preliminary data indicate that the external domain of sulfated CD44v$_{3,8-10}$ (Fig. 2C) in Met-1 cells preferentially binds vascular epithelial growth factor (VEGF), but not bFGF (data not shown). VEGF is a specific mitogen for endothelial cells and a potent microvascular permeability factor (Folkman, 1985; Dvorak et al., 1995). It plays an integral role in angiogenesis and thus in potentiation of solid tumor growth (Folkman, 1985; Dvorak et al., 1995). Recent data indicate that Met-1 cells are capable of inducing a high level of intratumoral microvessel formation (Young et al., 1995; Cheung et al., 1997). Therefore, the attachment of VEGF to the heparin sulfate sites on CD44v$_{3,8-10}$ may

be responsible for the onset of breast tumor-associated angiogenesis.

A number of different MMPs, including the 72-kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92-kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the stromelysins (MMP-3, MMP-11), and the interstitial fibroblast-type collagenase (MMP-1), are thought to play an important role in degrading ECM materials during tumor invasion and metastases (Merzak et al., 1994; Boyd, 1996; Chen, 1996). Biochemical interactions between MMPs and various cell surface molecules have not been fully established. A membrane-type MMP, or transmembrane MMP, and TIMP-2, a tissue inhibitor of MMP-2 have been reported to be involved in the activation of MMP-2 on the cell surface (Polette et al., 1996). Brooks et al. (1996) determined that MMP-2 is localized in a proteolytically active form on the surface of invasive cells based on its ability to bind directly to integrin αvβ3. The question of whether other MMPs are also involved in interacting with CD44-related adhesion molecules during the progression and metastasis of tumors remains to be answered.

In the present study, we used anti-CD44 or anti-MMP-9-mediated immunoblot/immunoprecipitation and gelatin zymography to demonstrate the possible physical association between these two molecules. Specifically, we found that $CD44v_{3,8-10}$ is closely associated with MMP-9 (gelatinase B) in the plasma membrane of Met-1 cells based on the evidence provided by anti-MMP-9 and anti-CD44-mediated immunoprecipitation followed by immunoblot and gelatin zymography (Figs. 1E, 4A-c). Furthermore, $CD44v_{3,8-10}$-associated MMP-9 appears to be present in a proteolytically active form (Fig. 4B-b) and preferentially localized at the "invadopodia" structure of the Met-1 cells (Fig. 5). Our results are consistent with previous findings showing that certain proteases are localized on "invadopodia" of human malignant melanoma cells (Chen, 1996). Therefore, we believe that the close interaction between $CD44v_{3,8-10}$ and the active form of MMP-9 in the "invadopodia" structure of Met-1 tumor cells may be required for the degradation of ECM for tumor cell invasion and metastasis. Preliminary data indicate that MMP-9 is complexed with the cytoplasmic domain (not the extracellular portion) of the $CD44V_{3,8-10}$ molecule (data not shown). The nature of the linkage between these two molecules is currently undergoing investigation.

The invasive phenotype of tumor cells characterized by "invadopodia" formation (Mueller and Chen, 1991; Monsky et al., 1994) and tumor cell motility (Jiang et al., 1994; Lauffenburger and Horwitz, 1996) has been linked to cytoskeletal function. Dissection of the transmembrane pathways controlling these cellular processes should aid in understanding the regulatory mechanisms underlying tumor invasion and metastasis. Previously, we have demonstrated that CD44 isoforms (e.g., CD44s and CD44v ex14/v10) display certain high affinity binding to the cytoskeletal protein ankyrin (Bourguignon et al., 1991, 1993, 1995; Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1994, 1996; Bourguignon, 1996). The cytoplasmic domain of the CD44 isoforms contain the ankyrin binding site (NGGNGTVEDRKPSEL), which is ≥90% conserved in all of the CD44 isoforms (Lokeshwar et al., 1994) and resides between amino acids 480 and 494 of the $CD44v_{3,8-10}$ molecule. This ankyrin binding sequence appears to be required for regulating CD44 isoform-mediated function (Bourguignon et al., 1995; Bourguignon, 1996). The fact that the synthetic peptide (NGGNGTVEDRKPSEL), which corresponds to the ankyrin binding domain of the CD44 isoform, competes effectively with $CD44v_{3,8-10}$ to bind ankyrin ($K_i \approx 0.7$ nM; Fig. 3) suggests that the sequence of NGGNGTVEDRKPSEL in the cytoplasmic domain of $CD44v_{3,8-10}$ interacts directly with ankyrin. Most importantly, we have found that treatments of Met-1 cells with certain agents including anti-$CD44v_{3,8-10}$, cytochalasin D (a microfilament inhibitor), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule-disrupting agent) effectively inhibit "invadopodia" formation and tumor cell migration (Table 2). Therefore, we believe that $CD44v_{3,8-10}$ plays an important role in linking ankyrin to the membrane-associated actomyosin contractile system required for "invadopodia" formation, tumor cell migration, and matrix degradation during breast cancer progression.

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APPENDIX - Z. Gunja-Smith
ITEM #2

ROLE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN HUMAN BREAST ADENOCARCINOMA.

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Excessive expression of a group of matrix metalloproteinases (MMPs) and or decreased production of their tissue inhibitors (TIMPs) have been implicated in tumor invasion and metastasis. In this study we have undertaken a unified multipronged approach to test this hypothesis by examining proteinases and inhibitor levels by zymography, immunohistochemistry and mRNA levels to show the expression or repression of MMPs and TIMPs in the breast tissues. Eighty breast tissue samples consisting of normal, benign tumors (fibroadenoma, tubular adenoma) and noninvasive (*in situ* ductal) or invasive (ductal and lobular) carcinomas were analyzed. The cancers were further classified as grades II or III. Specific MMPs (MMP-1 or 13, interstitial collagenase; MMP-2, (gelatinase A); MMP-9 (gelatinase B); MMP-7 (matrilysin); MT1-MMP (membrane type 1 MMP) and MMP-3 (stromelysin-1) together with specific TIMPs (TIMP-1, -2 and -3) were analyzed. Use of SDS-PAGE-gelatin or transferrin zymography identified and quantified (with an imager-software program) MMPs. MMP-1, MMP-2 ,-9 and MMP-3 were also quantified by tritiated-substrate assays. Reverse gelatin zymography and TIMP-1 ELISA kit were used to locate and quantify the TIMPs. Use of standard RT-PCR method was used to measure undegraded mRNA levels of some breast tissues. Using specific anti-MMPs and anti-TIMPs IgGs, immunohistochemical evaluations (staining score of 1-4) of sections of adjacent paraffin-embedded tissue blocks were carried out to locate and identify the expression of MMPs and TIMPs in breast pathology.

Keywords: **M**etalloproteinses (**MMPs**); **T**issue **I**nhibitors of **MMPs** (**TIMPs**); **G**elatinases; **I**mmunohistochemistry; **B**reast **T**umors.

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Extracts (0.25% Triton-X 100, 1 M GuHCL and 0.1% SDS in standard Tris buffer) of small amounts (100 -500 mg) of breast tissue analyzed by gelatin and transferrin zymography showed the presence of latent and active MMP-2 (72 kDa, 62 kDa) and MMP-9 (92 kDa, 82 kDa, 68 kDa)), higher molecular weight aggregates of MMP-2 and -9, serine proteinases and in some tissues MMP-1 (55 kDa). Biochemical quantification of MMPs showed an overall increase in all types of MMPs in cancer tissues. MMP-9 was the key MMP; it was present at levels 0.23 (*in situ* ductal) to 2.3 (infiltrating ductal) μg/g wet weight tissue in cancer tissues compared to unquantifiable amounts in normal (3/6) and benign neoplasm (11/16) tissues. There was a correlation of constitutively expressed MMP-1 (0.04 - 0.66 μg/g tissue) and MMP-2 (0.23 -1.7 μg/g tissue) in cancer tissues. Zymography also showed a fraction of MMP-9 and MMP-2 in their active forms in grade III breast cancers compared to normal and benign tissues.

Reverse zymography showed the presence of TIMPs -1,-2 and -3 in all breast tissues. Quantitation by TIMP-1 ELISA kit and reverse zymography clearly showed that total amounts of TIMPs were lower in cancer (0.2 and 1.0 μg/g wet weight tissue in grade III and II, respectively) compared to higher amounts in normal (4.5 μg) and benign (3.5μg) and noninvasive cancer (*in situ* ductal carcinoma, 1.5μg) tissues. The production of TIMPs in cancer tissues falls far short of the amount needed to counteract the excessive production of MMPs leading to an imbalance in enzyme and inhibitor expression.

Immunohistochemical evaluations of the adjacent paraffin-embedded tissue sections (3 μ) showed the direct production of MMP-9, MMP-2, MMP-1, MMP-7 and TIMP-1, -2 and -3 by various cell types; tumor cells or neighboring stromal cells. Sections of normal breast tissues showed the expression of MMPs and TIMPs mainly in the stromal component of the tissues with occasional staining of ductal epithelial cells. Only weak staining with anti-MMP-9 IgGs was seen in stroma. Normal tissues stained highly ($3\text{-}4^{+}$ staining) for TIMPs (stroma only) and stained 1^{+} for MMP-2 and MMP-1. Benign neoplasm sections exhibited the equal staining ($2\text{-}3^{+}$) of epithelial cells and stroma with MMP-2, MMP-1 and MMP-7 (weak stain) IgGs; stroma only with TIMP-1 and TIMP-2 IgGs (1^{+}); stroma and tumor cells with TIMP-3 IgGs and occasional staining (tumor cells) with MMP-9 IgGs. Cancer tissues of all grades and types showed staining with MMP-9 ($3\text{-}4^{+}$), MMP-2, MMP-1 and MT1-MMP IgGs. Weak to 1^{+} staining with TIMP-1, TIMP-2 and TIMP-3 IgGs was seen in stroma and in tumor cells. MMP-9 was found to be expressed (cytoplasmic staining) mainly by tumor (epithelial) cells.

This multipronged (biochemical and immunohistochemical) study categorically shows that there is an elevated expression of MMP-9 together with overexpression of constitutively expressed MMP-2 and MMP-1; and underexpression of TIMPs -1, -2 -3 by both stroma and tumor cells in all types of breast cancer. This imbalance of MMPs over their tissue inhibitors (TIMPs) may contribute to the invasiveness or metastatic potential of human breast cancer.

ROLE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN HUMAN ADENOCARCINOMA

Gunja-Smith[1], Z., Sittler[2], S.Y., Liu[1], Y., Woessner[3], J.F., Jr.,and Morales[2], A.R.

Department of Medicine[1], Department of Pathology[2], Department of Biochemistry and Molecular Biology[3], University of Miami School of Medicine, Miami, Fl 33101.

TYPE IN SELECT ABSTRACT: SINGLE SPACE 25 -WORD ABSTRACT BELOW THIS LINE. DO NOT LEAVE 1" SPACE BETWEEN AFFILIATION AND BEGINNING OF ABSTRACT TEXT. DO NOT EXCEED ONE PAGE.

The deadly consequences of breast cancer are largely due to metastasis, a process in which tumor cells penetrate the blood vessels and enter other tissues to spread the cancer. This movement through vessels and tissues is attributed to a group of digestive enzymes (the matrix metalloproteinases or MMPs) that can destroy the matrix in advance of tumor cell movement. These MMPs are normally produced in small amount and are held in check by inhibitors in the tissues (tissue inhibitors of MMPs = TIMPs). We took 80 samples of breast tissues (benign tumors and various carcinomas) and measured the production of six different MMPs and 3 TIMPs in a unified multipronged approach. We used antibody methods to see which cells are producing these enzymes and inhibitors. The most prominent enzyme was MMP-9, also known as gelatinase B, which is able to break down the wall that forms around tumor cell clusters. While this, and several other MMPs were elevated in cancer, the TIMP inhibitors were produced at levels well below normal. This results in an imbalance in which the destructive proteases greatly outweigh the controlling inhibitors, facilitating the spread of the cancer.

Zeenat Gunja-Smith

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APPENDIX - Z. Gunja-Smith
ITEM #3

The FASEB JOURNAL

ABSTRACTS

Satellite Meetings: P1-P344, M1-M56
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17th International Congress of Biochemistry
and Molecular Biology
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2333

PLASMINOGEN (PG) ACTIVATION BY TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) IS PROMOTED BY CYTOKERATIN 8 (CK8) ONLY IN A MULTIMERIZED STATE. K.R. Kralovich, L. Li, D.J. Webb, and S.L. Gonias, Univ. of Virginia, Charlottesville, VA, USA

CK8 is expressed on the surfaces of hepatocytes and breast cancer cells and released into the medium in the form of variably sized, heterogeneous polymers. CK8 binds Pg and t-PA, greatly accelerating the rate of Pg activation. To study these interactions in greater detail, a cDNA encoding 174 amino acids from the C-terminus of CK8 was expressed as a GST-fusion protein (CK8f). Purified CK8f bound ^{125}I-Pg and ^{125}I-t-PA in solution, as determined by the ability of CK8f to inhibit binding of these radioligands to immobilized CNBr-fragments of fibrinogen. ^{125}I-Pg and ^{125}I-t-PA also bound specifically to immobilized CK8f. Pg or t-PA, in nonradiolabeled form, completely inhibited the binding of ^{125}I-t-PA to immobilized CK8f and nonradiolabeled t-PA inhibited the binding of ^{125}I-Pg to immobilized CK8f; the K_i values for all four competitions were similar (0.5-1.3 μM). Thus, the binding sites for Pg and t-PA are apparently identical or overlapping. We hypothesized that a single CK8f could not bind Pg and t-PA simultaneously. To test this hypothesis, we studied the activation of Pg by t-PA in solution. In this system, soluble CK8f failed to promote Pg activation. By contrast, immobilized CK8f dramatically accelerated Pg activation. When 2 nM t-PA and 0.15-1.0 μM Pg were added to wells with immobilized CK8f, activation occurred almost exclusively in the immobilized phase. These results support a model in which Pg activation is promoted by the binding of Pg and t-PA to separate, but adjacent CK8f molecules. Similar interactions may also occur with intact CK8 on the cell surface or in polymers released from cells.

2334

TESTS NEEDED PRIOR THYROIDECTOMY IN THYROID FOLLICULAR CARCINOMA.

DR.QUSEY. G .O BATTIKHI,PH.D.,(BY INVITATION ONLY). MEDICAL TECHNOLOGICAL LAB'S FOR ANALYSES & RESEARCH, AMMAN-JORDAN.

Partial thyroidectomy was done on a 32 years old woman, due to apparently ,familial thyrotoxicosis,(loss of breath& irregular body - temperature), where 60 gms were removed from the left -lobe of the thyroid gland in 3 pieces.
After, partial thyroidectomy,histopathological tests ,have suggested a minimal follicullar carcinoma., & had recommended that sholud the left lobe of the thyroid gland ,have been completely removed no further surgery would have been required.

The patient,was put on 150 ug of thyroxine daily,for 3 months, afterwhich time, her serum:FT4=9.357 pmol/l, FT3=3.006 pmol/l,TSH=2.633 ulU/ml, Thyroglobulin= 25.385 ng/dl as thyroxine.
Her differential leucocytes count was: Neutrophils=16%, Lymphocytes=73%, Monocytes=10%,Eosinophils=1%,& Basophils=0%.

2335

PROMOTING ACTIVITY OF 2,5-DI(*TERT*-BUTYL)-1,4-HYDROQUINONE IN THE TRANSFORMATION OF BALB/3T3 CELLS AND ITS RELATIONSHIP TO THE INCREASE IN THE INTRACELLULAR FREE Ca^{2+} LEVEL. A. Sakai and R. Teshima, Natl. Inst. Health Sci., Setagaya-ku, Tokyo, Japan.

2,5-Di(*tert*-butyl)-1,4-hydroquinone (DTBHQ) is an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase and is reported to elevate the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in several cell culture systems. Thapsigargin has the same actions and is a tumor promoter. However, there has been no report so far concerning the enhancing effect of DTBHQ on carcinogenesis. We examined the promoting activity of DTBHQ in a two-stage transformation assay using BALB/3T3 cells. DTBHQ enhanced the transformation of cells pretreated by a sub-threshold dose of 3-methylcholanthrene in a dose-dependent manner but not in non-pretreated cells. Changes in $[Ca^{2+}]_i$ in response to DTBHQ were measured in fura-2-loaded BALB/3T3 cells using a digital imaging fluorescence microscopic analysis. DTBHQ induced rapid and sustained increases in $[Ca^{2+}]_i$ in a dose-dependent manner. The active range of the DTBHQ concentration in the Ca^{2+} response was in good agreement with that in the promoting activity of cell transformation (2.5–15 μM in the medium). An analog of DTBHQ, 2-*tert*-butyl-1,4-hydroquinone, did not enhance cell transformation nor elevate $[Ca^{2+}]_i$. These results suggest that the elevation of $[Ca^{2+}]_i$ may be involved in the promotion of BALB/3T3 cell transformation by DTBHQ.

2336

EXPRESSION OF GELATINASE B (MMP-9) IN MET-1 (INVASIVE, MOUSE) AND MCF-7 (HUMAN, NON-INVASIVE) BREAST CANCER CELL LINES, Z. Gunja-Smith, Y. Liu and L. Bourguignon, Univ. of Miami Medical School, Miami, Fl 33101, USA.

Gelatinase B (MMP-9, both latent and active forms) is constitutively secreted by mouse tumor cells (MET-1, high metastatic potential) in serum-free media. MMP-9 is also constitutively secreted (mainly in latent form) by the noninvasive human cell line MCF-7; these cells must be treated with phorbol ester (PMA) to secrete quantities of MMP-9 comparable to basal levels found in MET-1 cells. By use of monospecific antibodies (IgGs), confocal laser microscopy showed colocalization of MMP-9 and CD44 (a cell surface adhesion molecule);in particular, interaction with CD44 was prominent in the invadopodia of MET-1 cells. Immunoprecipitation of a membrane fraction with either monospecific antibody followed by gelatin zymography (to assay MMP-9) and immunoblot (CD44) confirms the close association of active MMP-9 and CD44 isoforms. However, latent MMP-9 was found to be closely associated with multiple CD44 isoforms in the resting and PMA-treated MCF-7 cells. These findings suggest that association of proteolytically active form of MMP-9 with CD44 isoforms of a surface adhesion molecule play a key role in rendering a cell line tumorigenic and ultimately of metastatic potential.
Supported by DOD grants DAMD 17-94-J-4295/4121 (ZGS/LB).

HEMATOLOGY (2337-2338)

2337

Alpha-thalassaemia and sickle cell haemoglobin in relation to infant anaemia and malaria

Chimsuku L[1], Brabin B[2], Verhoeff F[2], Broadhead R[1], Old J[3], Russell W[2]
[1] College of Medicine, Blantyre, Malawi, [2] Liverpool School of Tropical Medicine, Liverpool, [3] Institute of Molecular Medicine, John Radcliffe Hospital, Oxford

The objectives of this study were to (1) determine the prevalence of α$^-$-thalassaemia and sickle-cell haemoglobin in infants living in the Shire Valley, Malawi, an area with year round *P.falciparum* transmission, and (2) to assess their relationship with haematological indices and *P.falciparum* parasitaemia. Cord blood samples from 58 deliveries were screened for α$^-$-thalassaemia by Southern Blot analyses. 222 blood samples from infants older than 18 weeks were screened by Hb electrophoresis, for sickle-cell haemoglobin. The prevalence of $-\alpha^{3.7}\alpha\alpha$ was 41.0% and that of $-\alpha^{3.7}/-\alpha^{3.7}$, 8.7%. Hb SS occurred in 2.3% and HbAS in 18.1% of samples. Red cell counts were significantly higher and mean corpuscular haemoglobin significantly lower in infants with α-thalassaemia genotypes. There were no significant differences in mean Hb or other haematological parameters between infants with and without α$^-$-thalassaemia or sickle cell trait. *P.falciparum* parasitaemia was significantly more frequent throughout infancy in babies with HbAS. Older HbAS heterozygotes may have better immunological defence against *P.falciparum* infection because persistent parasitaemias in infancy stimulate host immunity.

2338

EFFECTS OF ANION TRANSPORT INHIBITORS ON HIGH-PRESSURE-INDUCED HEMOLYSIS IN HUMAN ERYTHROCYTES. Takeo Yamaguchi and Shigeyuki Terada, Fukuoka Univ., Fukuoka, Japan

When the erythrocyte suspension is subjected to a high pressure (0.1-200 MPa) for 30 min at 37 C, hemolysis starts to occur at about 130 MPa. At 200 MPa, the degree of hemolysis is a 40-50%. The effect of anion transport inhibitors on high-pressure-induced hemolysis was examined. The hemolysis at 200 MPa is suppressed by stilbene derivatives such as 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and 4, 4'-diisothiocyanodihydrostilbene-2,2'-disulfonate (H2-DIDS). The suppression of hemolysis is well correlated with the inhibition of anion transport, suggesting a contribution of band 3 to the membrane stabilization under high pressure. With incubating anion transport inhibitor-treated cells in alkaline pH, the suppressive effect decreases in the case of DIDS but not in H2-DIDS. At neutral pH, DIDS and H2-DIDS bind to Lys-539 of band 3. Upon exposure to alkaline pH of such cells, another NCS group of H2-DIDS reacts with Lys-851 of band 3. Therefore, different behavior of DIDS and H2-DIDS on hemolysis may be explicable in terms of the difference in the reactivity of band 3 with both inhibitors.

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EXPRESSION OF MATRIX METALLOPROTEINASE-9 (GELATINASE B) AND A TISSUE INHIBITOR OF METALLOPROTEINASE (TIMP-1) IN HUMAN BREAST ADENOCARCINOMA

Z. Gunja-Smith, Y. Liu, and S. Y. Sittler*. Departments of Medicine, and Pathology*, University of Miami Medical School, Miami, Fl 33101.

The deadly consequences of breast cancer are largely due to metastasis, a process in which tumor cells penetrate blood vessels and enter other tissues. This movement through vessels and tissues is attributed to matrix metalloproteinases [MMPs] that destroy the matrix. The small amounts of MMPs produced in normal tissues are held in check by tissue inhibitors, but increased production of MMPs or decreased production of the tissue inhibitors [TIMPs] may be implicated in tumor invasion and metastasis (1,2). In this study we used zymography, immunohistochemistry and mRNA assays to assess the levels of MMP-9 and TIMP-1 in the same sample of breast tissue. The different tissues analyzed included tissues that were biopsied as normal, or as benign [fibroadenoma] tumors, or as noninvasive [*in situ* ductal and *In situ* lobular] or as invasive [infiltrating or poorly differentiated ductal, lobular, medullary,and colloidal,] carcinomas.

Analysis of extracts [100 -150 mg] of breast tissue by gelatin and transferrin zymography (3) revealed the presence of latent [92 kDa] and active [82 kDa and 68 kDa] MMP-9 , latent [72 kDa] and active [62 kDa] MMP-2 and higher molecular weight aggregates of MMP-2 and -9. Quantification of MMP bands showed a general increase in MMPs in all cancer tissues. However, the increase in MMP-9 was the most striking, as it was present at levels 20 times [*in situ* ductal] to 200 times [infiltrating ductal] in carcinoma tissues compared to the low levels [often undetectable] found in normal tissues and benign tumors. Zymography also showed that a greater proportion of the latent MMP-9 and MMP-2 was present in their active forms in carcinoma tissues.

Reverse zymography (3) demonstrated the presence of TIMPs -1,-2 and -3 in all breast tissues, but quantitation of TIMP-1 [ELISA kit] clearly showed that the level of TIMP-1 was much lower in invasive cancer tissues compared to normal and benign tissues.

Immunohistochemical (4) staining of adjacent paraffin-embedded tissue sections (3 μ) with anti-MMP-9 or anti-TIMP-1 IgGs indicated that MMP-9 and TIMP-1 were produced by tumor cells and by stromal component of the breast tissue. Normal breast tissues showed the expression of

TIMP-1 [3 - 4^+ staining] in the stroma and occasional low staining for MMP-9 in epithelial cells. Benign neoplasm sections exhibited 2^+ staining for TIMP-1 in the stroma and occasional staining [epithelial cells] for MMP-9. Carcinoma tissues of all grades and types showed weak to 1^+ staining for TIMP-1 in stroma and strong staining for MMP-9 [3 - 4^+] in tumor cells. MMP-9 was found to be expressed [cytoplasmic staining] mainly in tumor cells.

Reverse transcription polymerase chain reaction [RT-PCR] analysis (5,6) was used as an assay for semi quantitation of low-abundance mRNAs of MMP-9 and TIMP-1 gene expression in breast tissues. Total RNA was isolated [GIBCO BRL kit] and 5'- and 3'- sequence-specific oligonucleotide primers were used to amplify 640 bp for MMP-9 cDNA, 551 bp for TIMP-1 cDNA and 230 bp for house keeping gene-GAPDH cDNA. The RT-PCR analysis showed a higher expression of MMP-9 in cancer tissues, the highest expression being noted in infiltrating and undifferentiated ductal carcinomas. In contrast, TIMP-1 expression was found to be lower in these carcinomas than in benign tissues .

This unified multipronged [biochemical, immunohistochemical and molecular biological techniques) study confirms that there is an elevated expression of MMP-9 and an underexpression of TIMP-1 in breast carcinoma tissues. In normal tissue, TIMP-1 has a dual role of complexing with latent MMP-9 to avoid its activation (7) and also to bind with the active enzyme and inhibit the enzyme action, thus limiting the degradation of the surrounding matrix. The observed imbalance of MMP-9 over tissue inhibitor (TIMP-1) in carcinoma tissue would lead to an increased matrix degradation and contribute to the invasiveness or metastatic potential of human breast carcinomas.

Supported by the grant from the U.S. Army Medical Research and Materiel Command under DAMD 17-94-4295.

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APPENDIX - Z. Gunja-Smith
ITEM #5

AMERICAN ASSOCIATION FOR CANCER RESEARCH

90th ANNUAL MEETING

APRIL 10-14, 1999 • PHILADELPHIA, PA

Volume 40 • March 1999

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Proceedings

#3108 MHC-restricted killing of N-myc amplified neuroblastoma tumor cells by N-myc peptide specific CTL. Asis K. Sarkar and Jed G. Nuchtern. *Baylor College of Medicine and Texas Children Cancer Center, Houston, TX 77030.*

The effectiveness of cell mediated immunotherapy of cancer can be limited by antigen loss mutations which occur during the growth of the tumor. Oncogene derived antigens may be better therapeutic targets because their expression is often required to maintain the malignant phenotype. Amplification of the N-myc oncogene is the most significant negative prognostic factor for children with advanced stage neuroblastoma. We propose that the N-myc protein would be an ideal target for vaccine therapy in these patients. We analyzed two 9 amino acid peptides from N-myc, both containing the HLA-A1 binding motif, for their ability to induce neuroblastoma specific cytotoxic T lymphocyte (CTL) lines. CTL were generated by in vitro stimulation of peripheral blood lymphocytes (PBL) from an HLA-A1 positive healthy donor using peptide-pulsed autologous PBL as antigen presenting cells. These CTL lines specifically lysed HLA-A1 positive, N-myc amplified neuroblastoma tumor cells but did not lyse either HLA-mismatched N-myc amplified neuroblastoma tumor cells or matched/non-matched non-amplified tumor cells. We generated similar neuroblastoma specific CTL by in vitro stimulation of PBL from an HLA-A1 patient with N-myc amplified neuroblastoma. The CTL activity was inhibited by W6/32 antibody but no inhibition was observed with antibody to HLA-DR (MHC-II) indicating the MHC-class I specificity of the CTL. The inhibition of CTL activity by anti-CD8 antibodies but not by anti-CD4 antibodies confirms the role of CD8+ CTL. This is the first report suggesting that N-myc derived antigenic peptides could be useful as part of a vaccine strategy for N-myc amplified neuroblastoma.

#3109 Molecular mimicry of CD55 by a human anti-idiotypic antibody 105AD7. Ian Spendlove, V. Potter, L.G. Durrant. *University of Nottingham, Academic Unit of Clinical Oncology, City Hospital, Hucknall Road, Nottingham, NG5 1PB.*

The antibody 791T/36 recognises CD55 (complement decay accelerating factor). The human anti-idiotype 105AD7 was cloned from a patient undergoing 791T/36 imaging for metastatic cancer. Both the antigen and anti-idiotypic antibody have been cloned and areas of homology between three CDRs and the first two repeat domains of CD55 have been identified. The mimicry of these regions was tested by 791T/36 binding to the individual peptides from both the CDRs L1, H2 and H3 of 105AD7 and the homologous peptides in CD55. The antibody was able to recognise each of the peptides individually and co-operative binding was seen when using combinations of peptides from either the anti-idiotype or the antigen. Confirmation of 105AD7 mimicry of CD55 was achieved using 105AD7 Ab3 sera that showed the same pattern of binding to peptides as 791T/36. Ab3 sera was tested on the individual peptides and showed a similar binding pattern to the 791T/36. This demonstrates the molecular mimicry of CD55 by 105AD7 is generated by three CDR regions that show amino acid and structural similarity to three regions of CD55 and that these are the contact points for 791T/36.

#3110 MHC class I processing of a misfolded transmembrane protein by a proteasome-independent pathway. Qin, J., Xu, Y., Moroi, Y., Setaluri, V. and Houghton, A.N. *Sloan-Kettering Division, Cornell University Graduate School of Medical Sciences, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.*

Transmembrane proteins that are translated in the endoplasmic reticulum (ER) can be processed for antigen presentation through several pathways, most frequently using proteasomes localized presumably in the cytosol. We examined processing of a chimeric mutant glycoprotein of a transmembrane tumor antigen from the tyrosinase family, gp75/tyrosinase-related protein 1, engineered to contain the K^b-restricted $OVA_{257-264}$ epitope in the ER lumenal domain. The chimeric gp75-OVA fusion protein was translated into the ER and co-translationally modified with asparagine-linked carbohydrate chains, but was misfolded and not able to egress the ER. The $OVA_{257-264}$ epitope was presented to $CD8^+$ cytotoxic T cells through the MHC class I pathway. Processing of $OVA_{257-264}$ was not affected by proteasome inhibitors lactacystin and LLnL. Processing and presentation occurred in RMA-S cells that have a deficiency in TAP-2, supporting presentation that was independent of TAP. However, processing could be effectively blocked by the serine protease inhibitor TLCK. These results are consistent with an alternative TLCK-sensitive processing pathway, independent of proteasomes and TAP transport, for antigen presentation of edited misfolded transmembrane proteins. The study shown here indicated a proteasome-independent pathway could be involved in processing misfolded transmembrane proteins, such as mutant proteins in cancer cells.

#3111 Signaling events involved in MMP-9 expression in T cells of mammary tumor bearing mice. Rivero, J.A., Gunja-Smith, Z., Alamino, M., Herbert, L., Charyulu, V.I., and Lopez, D.M. *Departments of Microbiology and Immunology and Medicine, University of Miami School of Medicine, Miami, Florida, 33101.*

Matrix metalloproteases(MMPs) are members of a multigene family of zinc endopeptidases which have been implicated in normal as well as various pathological conditions, including cancer invasion and metastasis. They are expressed in various cell types including lymphoreticular cells. Since the invasion of tumor masses by lymphocytes is known to be of crucial importance for the host defenses against the growing neoplasms, we evaluated the production of MMPs by the T lymphocytes from normal mice and tumor bearers. Using a murine mammary tumor model it was found by gel zymography that peripheral T lymphocytes from tumor bearing mice produce higher levels of MMP-9 than their normal counterparts. Moreover, since tyrosine kinases and PKC are proteins that are involved in signaling which may lead to gene regulation, we conducted studies using inhibitors to both in order to determine their involvement in MMP expression. Genestein, a tyrosine kinase inhibitor, induced a decrease in MMP-9 production while the use of staurosporine, a PKC inhibitor, increased MMP-9 levels. We studied the tyrosine phosphorylation pattern of T cells in normal versus tumor bearers and found that T cells from tumor bearers show a pronounced decrease in the levels of tyrosine phosphorylation of various proteins with disease progression. These results suggest that tumor burden may be affecting various signaling molecules which in turn may be the cause for the regulation of metalloproteases.

#3112 Combining co-stimulatory molecule B7.1-DNA with E7 peptide vaccination enhances the antigen's immunological effect. Khong, H., Gur, S., Chun, J., Allegra, C.J., Berzofsky, J.A., Khleif, S.N. *Medicine and Metabolism Branches, National Cancer Institute, Bethesda, MD.*

Effective antigen presentation to T cells requires two signals; the first signal is provided through the binding of T cell receptor (TCR) and the antigen/MHC complex. The second signal is mediated through the interaction of co-stimulatory molecules present on antigen carrying cells, such as B7 molecules and their receptors on the T cells. B7 proteins are transmembrane glycoproteins with two immunoglobulin-like extracellular domains, and are members of the immunoglobulin gene super family. There are at least two B7 molecules, B7.1 and B7.2. Although both of these molecules bind to CD28 and CTLA4 on the surface of T lymphocytes, they were found to have different effects. Vaccination of animals with cells expressing B7.1 along with HPV E7 significantly slows tumor growth when animals are challenged with tumors carrying the HPV E7 proteins. Here, we evaluated whether combining a DNA plasmid vector containing the ORF of the B7.1 with a specific antigenic peptide vaccination could enhance the immune response to the peptide. We found that the subcutaneous injection of B7.1 DNA prior to the injection of the E7 peptide significantly enhance the E7 specific CTL response. This effect was seen only if the injection of the B7.1 DNA and the E7 peptide was done in the same site but not if done in different sites. We optimized the schedule of such vaccination and we are in the process of correlating the effect with the expression and trafficking of the plasmid. In addition, we are also in the process of characterizing the mechanism and the influence of different cytokines on such vaccination. Since we have shown that we can combine a DNA construct with specific antigen-peptide vaccination, we believe that this could form a practical system to translate into the clinical setting.

#3113 MUC1-specific CTLs detected in human MUC1 transgenic mice (MUC1.Tg) that spontaneously develop pancreatic tumors. Mukherjee, P., Madsen, C., Canales, N., Sterner, C.J., Hollingsworth, M.A., and Gendler, S.J. *Mayo Clinic Scottsdale, AZ 85259; The Eppley Institute, Omaha, NE 68198 (M.A.H.).*

The MUC1 antigen is a large mucin glycoprotein that is overexpressed in human breast, pancreatic and other adenocarcinomas. Increased levels of MUC1 expression combined with decreased glycosylation, as well as the presentation of previously sequestered immunodominant epitopes, make this protein an attractive target for cancer immunotherapy. Suitable preclinical immunotherapy studies require adenocarcinoma models expressing human MUC1 protein. We developed a double transgenic mouse (designated MET) by crossing MUC1.Tg mice with mice that express the first 127aa of SV40 large T antigen under the control of the rat elastase promoter. We characterized tumor development in the MET mice. Immunohistochemical analysis of the pancreatic tumors using antibody to human MUC1 showed that the tumors expressed large amounts of MUC1 protein. We found that unimmunized MET mice develop MUC1-specific CTLs, killing 30% of the targets (B16 melanoma transfected with MUC1) by week 12; killing increased to 80% by week 18. The profile of killing was different using another target, C57MG mammary tumor cells expressing MUC1. Twenty percent killing was detected at week 12 which increased to about 30% by week 20. No CTL activity was detected when a MUC1 expressing pancreatic tumor cell line (Panc 02) was used as a target. The MET mice are on a C57 B1/6 background, which express $H\text{-}2K^b$ /$H\text{-}2D^b$. High levels of $H\text{-}2K^b$ /$H\text{-}2D^b$ class I molecules were detected on the B16 melanoma and the C57MG cells but not on the Panc 02 cells. The MUC1-specific MHC restriction properties of the CTL are under investigation. The activity of these cells *in vivo* remains to be determined, since the tumors grow progressively in these mice.

#3114 Expression of MUC1 mucin on activated T-cells from human MUC1 transgenic mice (MUC1.Tg): Implications in the development of MUC1 based vaccines for cancer. Mukherjee, P., Canales, N., Sterner, C.J., and Gendler, S.J. *Mayo Clinic Scottsdale, AZ 85259.*

Epithelial cell associated mucin, MUC1, is a large transmembrane glycoprotein that is expressed on many human epithelial tumors. In cancer, MUC1 is overexpressed and less glycosylated, thus exposing the immunodominant peptide epitopes that are normally hidden by oligosaccharides. This aberrant expression of MUC1 makes it an attractive target for cancer immunotherapy. It has been recently reported that MUC1 is expressed on normal activated human T-cells,